Non-thermal Inactivation of Vegetative Bacteria: Kinetics, Methods and Mechanisms

by

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Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

University of Tasmania, December, 2008

DECLARATION

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution, and to the best of my knowledge contains no copy or paraphrase of material previously published or written by any other person except where due reference is made in the text of the thesis.

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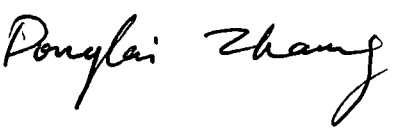

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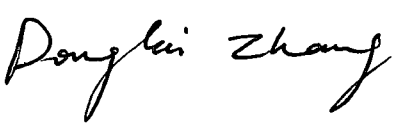

LIST OF PUBLICATIONS

T. Ross, D. L. Zhang and O. McQuestin (2008) Temperature is the main factor governing the rate of non-thermal inactivation of vegetative bacteria" *International Journal of Food Microbiology* 128: 129-135

D. L. Zhang, O. McQuestin, L. Mellefont and T. Ross (2008) The influence of non-lethal temperature on the rate of inactivation of vegetative bacteria in inimical environments is not species-dependent. *Food Microbiology* (accepted subject to revision)

D. L. Zhang, S. A. McCammon, J. P. Bowman and T. Ross (2008) Elongation Factor EF-Tu as an Indicator of Cell Viability. *Microbiology* (publications in preparation)

D. L. Zhang, J. P. Bowman and T. Ross (2008) A time-course study of genomic analysis on the responding of *L. monocytogenes* to Low pH and Low a_w *Applied and Environmental Microbiology* (publications in preparation)

STATEMENT OF AUTHORSHIP

The following people contributed to the publication of work undertaken as part of this thesis:

1. Ross *et al.*, 2008 (published in *International Journal of Food Microbiology)*

T. Ross: 50 %, Candidate: 20%, O. McQuestin: 30 %

The candidate principally contributed laboratory investigations and data analysis. O. McQuestin and T. Ross were involved in experimental design, data analysis and manuscript preparation.

2. Zhang et al., 2008 (accepted subject to revision by *Food Microbiology*)

Candidate: 60%, O. McQuestin: 15 %, L. Mellefont: 5 %, and T. Ross: 20%

The candidate undertook almost all laboratory investigations, and contributed to experimental design, data analysis, and manuscript preparation. O. McQuestin contributed to manuscript preparation and data analysis. L. Mellefont contributed to experimental design. T. Ross contributed to experimental design, data analysis and manuscript preparation.

3. Zhang et al., 2008 (publications in preparation)

Candidate: 55%, S.A. McCammon: 10%, J.P. Bowman: 20 % and T. Ross: 15 %

The candidate contributed to experimental design, laboratory investigations, data analysis, and preparation of the manuscript. S.A. McCammon contributed laboratory assistance. J.P. Bowman and T. Ross contributed to experimental plan, data analysis and manuscript preparation.

4. Zhang et al., 2008 (publications in preparation)

Candidate: 65%, J.P. Bowman: 20% and T. Ross: 15%

The candidate contributed to experimental design, laboratory investigations, data analysis, and manuscript preparation. J.P. Bowman and T. Ross contributed to experimental plan, data analysis and manuscript preparation.

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ABSTRACT

The inactivation of microorganisms with treatments of temperature, pH and water activity is of vital importance to the food industry. In a previous study in fermented meat products (Ross et al., 2004), it was observed that temperature is the dominant factor governing the rate of inactivation of *E. coli*. This study investigates whether this observation is true for other microorganisms and the mechanisms behind the phenomena. To better characterize and understand non-thermal inactivation, this dissertation involves three main phases: kinetics investigation between two species, *E. coli* and *L. monocytogenes*, methods development (luminometry) and studies on mechanisms of non-thermal inactivation of *L. monocytogenes*, including quantitative real time PCR (QPCR) and microarrays analysis.

To study non-thermal inactivation kinetics, *Listeria monocytogenes*, a foodborne bacterial pathogen with different characteristics to *E. coli*, was selected in this dissertation. Specifically, sixty-three inactivation rates were determined for both species at a non-growth-permissive pH and water activity (pH 3.50 and a_w 0.90 respectively) at nine growth permissive temperatures. The results showed that inactivation rates of both species were very similar, and the inactivation rate responses of both were comparable to those previously and independently reported for a variety of *E. coli* strains under a wide range of growth-preventing pH and water activity conditions (McQuestin, 2006). Thus, it appears that the influence of non-lethal temperature on the rate of inactivation of vegetative bacteria in inimical environments is not species-dependent.

For methodology, luminometry, in which intracellular ATP level is measured by its ability to generate light using the luciferin/luciferase enzyme system has been investigated as means of quantifying microbial loads. To assess luminometry as a more rapid method of enumeration of bacteria in inimical environments, exponential phase *L. monocytogenes* ScottA and Fw 03/0035 were inactivated under inimical conditions (pH 3.50 and a_w 0.90) at 25°C, 35°C and 45°C. Samples were periodically withdrawn for parallel viable count and luminometric analysis. The results showed that inactivation rates and kinetics determined by the ATP method were not comparable to those from viable counts. However, when both methods were applied to conditions permitting cell growth, there was a good correlation. Thus, the ATP method is not sensitive enough to quantify microbial inactivation, particularly when cells are inactivated in sub-lethal conditions; but it is well correlated with microbial growth.

To better understand the physiology of bacterial cells in inimical environments, particularly whether they are metabolically active, *tuf* gene expression was studied using QPCR methods. *L. monocytogenes* strains ScottA and Fw 03/0035 were inactivated with the same conditions as that described for the luminometry experiments. Although viable cells numbers decreased from 10^8 to less than the detection level (1.3×10^1 cells ml^{-1}), the *tuf* gene mRNA level remained unchanged. To determine whether this relatively high level of mRNA was due to unexpected stability of the mRNA or due to *de novo* synthesis, additional experiments were undertaken. Cells were inactivated under either mildly lethal temperature (55°C), in the presence of rifampin (which inhibits DNA-dependent RNA polymerase) or a

combination of both. The results show that when the antibiotic was present *tuf* gene expression was reduced much more completely, with a three log reduction compared with mildly lethal temperature with higher *tuf* gene levels of only a half log reduction. This raises the possibility that *L. monocytogenes* under mildly lethal conditions of pH and a_w or high temperature retain viability after being rendered non-culturable.

To explore the genetic responses to the inactivation phenomena observed, genomic microarray analysis was performed to determine the effects of low pH (3.5) and low a_w (0.90) on exponential phase *L. monocytogenes* ScottA, in a time-course experiment (5 min, 24 h, 48 h, 72 h). The results suggest that a large number of genes relevant to amino acid biosynthesis and metabolism are up-regulated, indicating a possible switch to alternative carbon sources as an energy supply and aid to maintenance of cell integrity. Genes belonging to the categories of structure and function of cell wall, cell movements, and carbohydrate metabolism were down regulated indicating lowered mobility. The regulatory network might play an important role in regulating cellular physiological status and may dictate the rate of inactivation.

In this dissertation, the hypothesis that temperature is the main factor governing the rate of inactivation of vegetative bacteria was firstly investigated and suggested to be non-species dependent and this will be very useful to understand microbiological safety of non-thermal processed food. For physiology of non-thermal inactivation, the thesis is fully addressed that when cells encounter environmental stresses, the

regulatory network might play an important role in up-regulating and down-regulating house keeping genes to cope with sublethal conditions and that when they reach the point of completely losing their culturability, they may still remain viable, thus entering the state of viable, but non-culturable, cells.

ACKNOWLEDGEMENTS

It has been a journey for me to start as a Master student and end up holding a Ph.D degree in only three years at the University of Tasmania, where I mixed in a totally different culture and joined a nice group of people, without whom I would not have accomplished such an amazing progress in my life.

Firstly, I would like to give my sincere thanks to Associate Professor Tom Ross for his great support on my project, both academically and financially. His guidance is such a great help for me on undertaking Ph.D studies and for completion of the dissertation.

Dr. Lyndal Mellenfont would be the second person I would like to thank. She is more likely to be a nice friend. Especially her supportive messages before my presentation always give me strength to give a good talk.

Associate Professor John Bowman, as my molecular research supervisor, gave me a lot of advice on genetic interpretation of QPCR and Microarray results.

Professor June Olley for her time and thoughts on proof reading of the thesis.

Students and staff from the Food Safety Centre and School of Agricultural Science, particularly:

Dr Olivia J. McQuestin for the method of kinetics studies and proof reading of a paper;

Ms Sharee A. McCammon for helping with QPCR method development;

Ms Esta Kokkoris for helping with overnight experiments of kinetics studies;

Ms Julia Souprounova for the method of RNA isolation on Microarray studies;

Dr Judith-Anne Marshall for helping with the method on ATP studies;

Ms Ann Gaffney for her sincere friendship, and

Dr Jiayin Pang for her friendship and helping on computer skills.

I would like to give my last sincerest thanks to my parents for their endless support and love.

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CHAPTER 1

LITERATURE REVIEW

1.1 FOOD MICROBIOLOGY

Food is an issue all over the world and has been all through history. However, it was not until the 19th century that food microbiology became a science (Brock and Madigan, 1991). In 1837, from the problem of souring of milk, Louis Pasteur, who was the first person to appreciate and understand the presence and role of microorganisms in food (Jay, 1992), discovered that certain bacteria were associated with food spoilage and that others caused specific diseases.

The field of food microbiology is among the most diverse of the areas of study within the discipline of microbiology. Its scope encompasses a wide variety of microorganisms including spoilage, probiotic, fermentative, and pathogenic bacteria, molds, yeasts, viruses, and parasites; a diverse composition of food; a broad spectrum of environmental factors that influence microbial survival and growth; and a multitude of research approaches that range from very applied studies of survival and growth of foodborne microorganisms to basic studies of the mechanisms of pathogenicity of harmful foodborne microorganisms (Mossel *et al.*, 1991).

1.2 FOODBORNE DISEASES AND PATHOGENS

In recent decades, the burden of foodborne disease has increased worldwide and the pattern of foodborne disease has changed substantially. Many of the foodborne pathogens that are of concern today were not even recognized as causes of

foodborne illness just 20 years ago (Desmarchelier and Grau, 1997). Since widespread food distribution and changes in methods of food preparation, outbreaks are more likely to be far reaching, and some are even global in scale (Rossi et al., 2008). For example, one of the worst food poisoning incidents in the history of the United States occurred in 1985 when 16,284 cases and 7 deaths were documented when pasteurized milk somehow became recontaminated with a potent strain of *Salmonella* Typhimurium. In 1994, this was exceeded by a national outbreak of *Salmonella* Enteritidis affecting 225,000 people who consumed contaminated ice cream products (Hennessy *et al.*, 1996).

Not only is the burden of foodborne disease increasing, but the nature of food poisoning is also changing. With the emergence of foodborne organisms with a low infective dose, such as entero-pathogenic *Escherichia coli*, food poisoning cases are now often of a sporadic nature and only detectable through surveillance (Hall et al., 2002, Sharma et al., 2008).

1.3 ESCHERICHIA COLI

Escherichia coli, the Gram-negative bacterium, which was originally named as *Bacillus coli*, was first described in 1885 by Dr. Theodor Escherich (Escherich, 1989). At the time, microbiological standards and regulations were being formulated, and it was realized that tests could not be conducted for each and every enteric pathogen that might be present in a sample. Instead, an indicator of potential contamination must be selected. In 1892, F. Schardinger suggested that *Escherichia coli* would be useful as an indicator of faecal pollution (Doyle *et al.*, 1997). Since then, it has become among the most extensively studied bacteria (Dobson, 2006).

E. coli were initially considered to be a harmless commensal. In the 1900s, *E. coli* was recognized as pathogenic when it was associated with white scours in calves (Doyle and Padhye, 1989). Studies in the 1920s and 1930s suggested that a number of strains could cause diarrhoea in infants; however, it was not until 1940s that a study clearly demonstrated the ability of some strains of *E. coli* to cause diarrhoea in humans. Since then the concept of *E. coli* as a cause of human diarrhoea was accepted (Doyle and Padhye, 1989). Now, pathogenic *E. coli* are known to cause a variety of clinical illnesses (Sharma et al., 2008, Dobson, 2006, Alexandre and Prado, 2003).

However, it was not until 1982, that some *E. coli* strains were first recognized as serious foodborne pathogens, after two foodborne-associated outbreaks. The outbreaks were caused by *E. coli* O157:H7 which was associated with undercooked ground beef in the USA (Riley *et al.*, 1983). Since then, numerous outbreaks of *E. coli* infection associated with foods have been reported (Bettelheim, 2000, WHO, 1997, Willshaw et al., 1994, Dobson, 2006).

1.3.1 Classification

E. coli is a member of the family *Enterobacteriaceae*. It is a Gram-negative straight rod, oxidase negative, motile by peritrichous flagella, or nonmotile, and facultatively anaerobic (Ørskov, 1984). They are a common part of the normal facultative anaerobic microbiota of the intestinal tracts of humans and warm-blooded animals.

The ability to distinguish strains of *E. coli* serologically was important in early studies. Serotyping is based on the many antigenic differences found in structures on the bacterial surface. The three fundamental antigens are O (somatic), K (capsule), and H (flagella). At present, a total of more than 170 different O groups, more than 80 K antigens and over 50 H antigens have been recognized (Desmarchelier and Grau, 1997).

One characteristic that distinguishes pathogenic from non-pathogenic *E. coli* is that the pathogenic strains can colonise sites in the body where nonpathogenic *E. coli* normally do not survive (Evans and Evans, 1983, Vallance et al., 2002). According to their pathogenicity and clinical features of the infections caused pathogenic *E. coli* strains are classified into nine groups (Desmarchelier and Grau, 1997): enteroinvasive *E. coli* strains (EIEC), enterotoxigenic *E. coli* strains (ETEC), enteropathogenic *E. coli* strains (EPEC), enteroaggegative *E. coli* strains (EAEC), diffuse-adhering *E. coli* strains (DAEC), necrotoxigenic *E. coli* (NTEC), cytolethal distending toxins *E. coli* (CDT), Shiga toxin-producing *E. coli* strains (STEC) and enterohemorrhagic *E. coli* (EHEC).

1.3.2 Pathogenicity

Pathogenic *E. coli* can be divided into two classes of infection: extraintestinal infections including neonatal meningitis, septicaemia and urinary tract infections (Ørskov, 1984, Dobrindt, 2005), and gastrointestinal infections (Buchanan and Doyle, 1997), which ranges from mild diarrhoea to a number of potentially fatal syndromes. Diarrhoeagenic *E. coli* are the specific cause of gastrointestinal infections and were recently identified as the main cause of foodborne

gastroenteritis in Australia, causing an estimated 563 000 cases annually (Hall *et al.*, 2005).

The precise mechanism of pathogenicity of EHEC (e.g. *E. coli* O157:H7) has not been fully elucidated. However, substantial progress in characterizing adherence factors and their genes has been made recently (Spears et al., 2006, Welinder-Olsson and Kaijser, 2005, Vallance et al., 2002, Joseph et al., 2002). The AE lesion is characterized by intimate attachment of the bacteria to intestinal cells, with effacement of the underlying microvilli and accumulation of filamentous actin in the subjacent cytoplasm (Obrien *et al.*, 1992). EHEC had been considered for many years a non-invasive bacterium. However, Oelschlaeger et al (1994) observed that unlike *Shigella* and *Salmonella* strains, which rely on microfilaments to invade human cells, *E. coli* EHEC strains rely on microtubules for invasion.

EHEC produces two cytotoxins that are cytotoxic to Vero cells (an African green monkey kidney cell line) and thus were originally named verotoxin 1 (VT1) and verotoxin 2 (VT2) (Konowalchuk et al., 1977, Welinder-Olsson and Kaijser, 2005). The toxins enter the bloodstream and bind to receptors, globoseries glycolipids on the eukaryotic cell surface, and that are most prevalent on renal cells, to directly cause disease.

The possible mechanism of EHEC pathogenesis (Spears et al., 2006, O'Brien et al., 1992, Welinder-Olsson and Kaijser, 2005) is that, after EHEC cells are ingested, the bacteria colonize the large intestine and adhere to and possibly invade colonic mucosal epithelial cells, replicate and destroy colonic cells, and damage the

underlying tissue and vasculature, possibly by both exotoxin-related and endotoxin-related mechanisms, thereby producing bloody diarrhoea with severe abdominal pain.

1.3.3 Susceptible Populations and Toxic Infective Dose

All age groups can be affected by EHEC, but infants, young children, the elderly and the immunocompromised most frequently experience severe illness (Welinder-Olsson and Kaijser, 2005). There are two complications of haemolytic colitis: haemolytic uraemic syndrome (HUS), usually occurring in children, and thrombotic thrombocytopeanic purpura (TTP), principally occurring in adults, both of which are potentially fatal diseases (Buchanan and Doyle, 1997, Noel and Boedeker, 1997, Welinder-Olsson and Kaijser, 2005, Karch et al., 2005).

Retrospective analysis of food associated outbreaks revealed that the infective dose of EHEC infection is quite low. For example, between 0.3 to 0.4 *E. coli* O157: H7 cells per gram were detected in several intact packages of salami that were associated with a foodborne outbreak (Doyle et al., 1997). This suggests that the infectious dose of *E. coli* O157: H7 is less than a few hundred cells (Grif et al., 1998). In some cases, the consumption of less than 50 cells may cause infection (Tilden et al., 1996).

Another food-borne bacterium that has caused severe outbreaks in recent decades, and typically involving a high mortality rate, is *Listeria monocytogenes*. While very different to *E. coli*, both in terms of ecology and physiology, both of these bacteria require more research to devise better control strategies to safeguard public health.

1.4 LISTERIA MONOCYTOGENES

The first confirmed diagnosis of *Listeria monocytogenes* in a human was that of a soldier suffering from meningitis at the end of the First World War. Two years later, Murray et al (1926) wrote the first published description of *Listeria monocytogenes*. It was not until 1940 that the identity of the organism was clear, and this new bacterium was named *Listeria monocytogenes*, a short, motile, Gram-positive, non-sporeforming rod that exhibits characteristic Gram-positive cell wall structure by electron microscopy (Kathariou, 2002, Rossi et al., 2008, Khelef et al., 2006, Sutherland et al., 2003).

1.4.1 Classification

The genus *Listeria* belongs to one of the *Clostridium* subbranches together with *Staphylococcus, Streptococcus, Lactobacillus* and *Brochothrix* (Sutherland et al., 2003, Khelef et al., 2006). This phylogenetic position of *Listeria* is consistent with its low G+C DNA content (36-42%) (Seeliger and Jones, 1986).

As for the phylogenetic analysis of *Listeria,* introduction of molecular biology methods allowed a better appreciation of the diversity within the genus *Listeria* which now contains only six species: *L. monocytogenes, L. innocua, L. seeligeri, L. welshimeri, L. ivanovii* and *L. grayi*. Only *L. monocytogenes* and *L. ivanovii* are considered to be virulent, with respect to both the 50% lethal dose in mice and the ability to grow in mouse spleen and liver (Jay, 1992, Jay, 2000, Khelef et al., 2006). Only one species, *L. monocytogenes*, is a human pathogen of high public health concern (Rossi et al., 2008).

Until recently, *L. monocytogenes* typing, which is used to characterized strains beyond the species level, relied mainly on serotyping and phage typing. There are 13 serovars of *L. monocytogenes*: 1/2 a, 1/2 b, 1/2 c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7. However, 95% of human isolates belong to three serovars: 1/2 a, 1/2 b, 4b (Sutherland et al., 2003, Swaminathen, 2001, Khelef et al., 2006).

During the 1980s, discovery of several listeriosis outbreaks that were positively linked to consumption of cheese and raw vegetables has led to inclusion of *L. monocytogenes* in the current list of bonafide foodborne pathogens. From that time on, serovar 4b strains have been responsible for 33 to 50% of sporadic human cases of all major foodborne listeriosis outbreaks worldwide (Doyle et al., 1997, Rossi et al., 2008).

1.4.2 Pathogenicity

Many methods for studying *L. monocytogenes* pathogenicity have been developed, including tissue culture assays and tests using laboratory animals. Considerable heterogeneity in levels of virulence of various *L. monocytogenes* strains has been observed (Swaminathen, 2001). Although rare nonpathogenic or weakly pathogenic *L. monocytogenes* isolates have been reported, all strains of the species are considered to be potentially capable of causing human disease (Rossi et al., 2008).

L. monocytogenes crosses the intestinal barrier in animals infected by the oral route. Bacteria are then internalized by resident macrophages, in which they can survive and replicate (Jay, 2000, Kathariou, 2002). They are subsequently transported *via* the blood to regional lymph nodes. Depending on the level of T-cell response

induced in the first days following initial infection, further dissemination *via* the blood to the brain or, in the pregnant animal, the placenta, may subsequently occur (Swaminathen, 2001). Hence, infection is not localized at the site of entry but involves entry and multiplication in a wide variety of cell types and tissues. In vitro, *L. monocytogenes*, invades many cell lines of different types (macrophages, fibroblasts, hepatocytes, and epithelial cells) (Portnoy *et al.*, 2002) and is one of the most invasive bacteria known.

As an intracellular pathogen, the spread of *L. monocytogenes* from cell to cell occurs without having to leave the inner parts of host cells, and allows them to disseminate within host tissues and induce the formation of infectious foci while being sheltered from host defenses such as circulating antibodies (Jay, 1992, Kathariou, 2002). *L. monocytogenes* first enter cells by phagocytosis after attaching *via* a surface-bound bacterial protein, called internalins, which are required for entry into epithelial cells and encoded by genes, e.g. *inlA* and/or *inlB* (Jay, 2000). *L. monocytogenes* is internalized in membrane-bound vacuoles. The main factor involved in lysis of the vacuole is a bacterial enzyme listeriolysin O (LLO), encoded by gene *hly* that has a pore-forming activity (Carrero et al., 2008). However, a second factor, a phosphatidylinositol-specific phospholipase C (PI-PLC), may also be involved (Erdenlig et al., 2000). Once escaping from the vacuole, *L. monocytogenes* rapidly accesses the cytosol, where they multiply (Portnoy *et al.*, 2002). Actin polymerization is essential to intracellular movement and cell-to-cell spread, and requires expression of the *actA* gene to form ActA surface protein to form actin tails, which propel the organisms toward the cytoplasmic membrane. The actin "comet tail" is made of actin microfilaments that are continuously assembled

in the vicinity of the bacterium. The actin comet tail is stationary in the cytosol and left behind by moving bacteria. The length of the tail is thus proportional to the speed of movement, with faster-moving bacteria having longer tails (Tilney and Portnoy, 1989, Gouin et al., 1999). When bacteria reach the plasma membrane, they force the cell to put out long protrusions with bacteria in the tips, which then to form double membrane vacuoles by an adjacent cell's internalization. After lysis of this vacuole, a new cycle of growth and mobility begins (Swaminathen, 2001). The entire cycle is completed in about 5 hours.

1.4.3 Listeriosis

Listeriosis is an atypical foodborne disease because of the severity and nonenteric nature, a high case-fatality rate, a frequently long incubation time, and a predilection for individuals who have underlying conditions that involve impairment of T-cell-mediated immunity (Jay, 2000, Lecuit, 2007, Seveau et al., 2007).

Listeriosis in humans is characterized by a variety of severe syndromes. The high-risk group includes the elderly, pregnant women, newborns and immuno-compromised individuals. Pregnant women are most frequently affected in the third trimester. The infection of the mother may be characterized by a flu-like illness, but the pathogen can cross the placental barrier and infect the unborn child, resulting in spontaneous abortion, foetal death, or stillbirth. In non-pregnant adults, *L. monocytogenes* primarily causes septicemia, meningitis, and meningoencephalitis, but it also can cause mild gastrointestinal illness, which is often associated with ingestion of high doses of organisms by healthy individual (Seeliger and Jones,

1986, Lecuit, 2007). The overall case-fatality rate for systemic or invasive listeriosis is usually about 20 to 30% for both epidemic and sporadic cases, however, the perinatal and neonatal mortality rate can be as high as 80% (Smith *et al.*, 2003).

During the last two decades, Listeriosis has emerged as one of the major foodborne diseases, because *L. monocytogenes* is ubiquitous, is resistant to diverse environmental conditions such as low pH and high NaCl concentrations, and is microaerobic and psychrotrophic (Sutherland et al., 2003, Lecuit, 2007). Many of the traditional preservative systems used in foods, alone or in combination, have little effect on the multiplication of this organism. In addition, the usual incubation time ranges from one day for non-invasive listeriosis up to several weeks for invasive listeriosis (Jay, 1992). So, the absence of definitive data regarding the infectious dose has prompted several countries to declare zero-tolerance (in 25g samples) for *Listeria* in certain products (Jay, 2000, Swaminathen, 2001). The annual incidence in Australia, similar to most industrialized nations, is between 2 and 7 cases per million population (Hall *et al.*, 2005) and has remained steady over the past 10 years (Sutherland et al., 2003). Due to these factors, *L. monocytogenes* remains a food-borne health hazard (Rossi et al., 2008).

1.5 MICROBIAL POPULATION GROWTH AND INACTIVATION

1.5.1 First Order Kinetics

Growth is defined as an increase in the number of microbial cells in a population, which can also be measured as an increase in microbial mass. Growth rate is the change in cell number or mass per unit time. During the cell-division cycle, all the structural components of the cell double. The interval for the formation of two cells

from one is called a generation, and the time required for this to occur is called the generation time. Thus the generation time is the time required for the cell number to double (Monod, 1949).

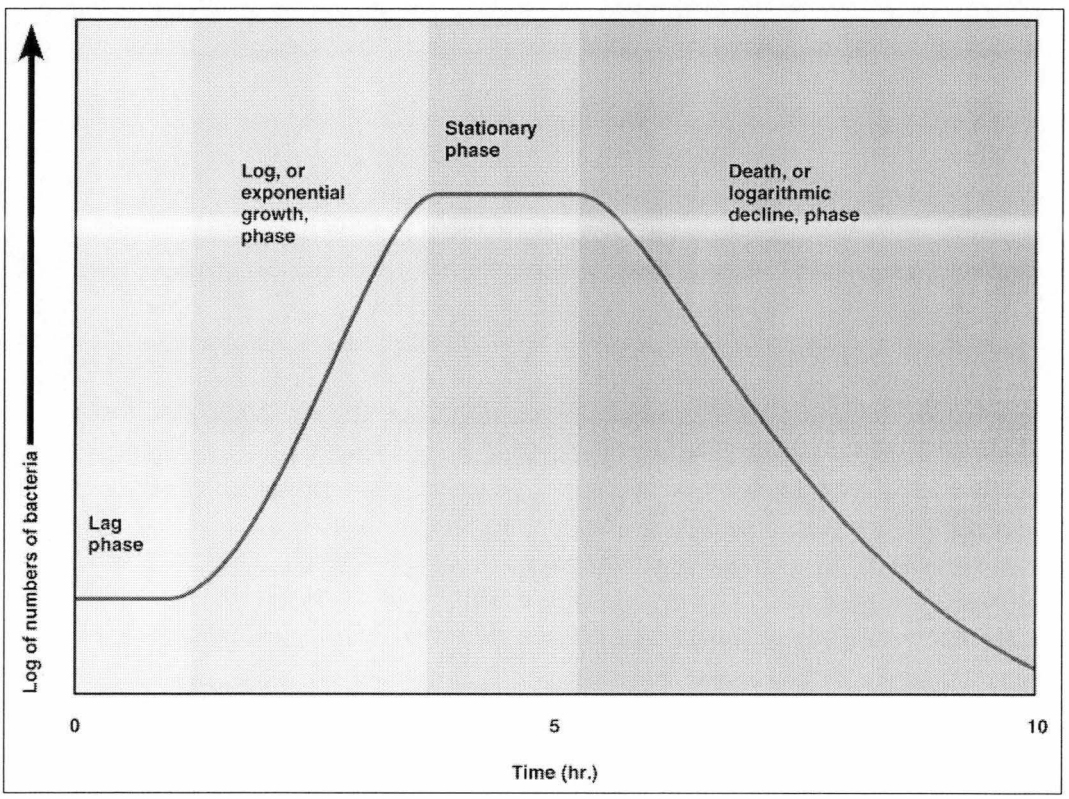


Fig 1.1 Typical growth curve for a bacterial population, including Lag phase, Exponential phase or Log phase and stationary phase (Death or inactivation phase see Fig 1.2 for details). The arrow indicates the direction of increasing of values of log numbers of bacteria (reproduced from http://diverge.hunter.cuny.edu/~weigang/Lecture-syllabus.html).

The typical growth curve for a population of cells is illustrated in Fig 1.1. This growth curve can be divided into three phases: the lag phase, which is a period of time after a microbial population is inoculated into a fresh medium and before the exponential growth phase. It is a consequence of the fact that each cell divides to

form two cells, each of which also divided to form two more cells, and so on. Stationary phase follows, when exponential growth ceases, and there is no net increase nor decrease in cell number.

Probably due to an essential nutrient of the culture medium being used up or build up of some waste product of the organism in the medium to an inhibitory level, when incubation continues after a population reaches the stationary phase, the cells may remain alive and continue to metabolise, but often they die, leading to a decrease in the viable count of the culture with a concurrent drop in the direct microscopic count (McQuestin, 2006).

When a bacterial cell is described as "viable", it means, it can grow to detectable levels if given a favourable environment. When a microorganism is unable to do so, it is considered non-viable, or dead. The term "viability" is used to describe the proportion of the bacterial population that is viable. While, the term "inactivation" is employed to describe irreversible loss of viability (Brown, 2002).

In the early 1900's the kinetics of microbial inactivation were described: the logarithm of the number of viable cells exposed to an inimical environment declined linearly as a function of time, and suggested that log-linear inactivation could be universally applied to the death of microbial populations in response to any environmental parameters, as Fig 1.2 A shows (Withell, 1942). The decimal reduction time (D-value) is the time taken to reduce the population by 90%. This first-order reaction, presumed to be caused by the inactivation of one critical site per cell (Humpheson et al., 1998), is described mathematically as $Nt = N_o e^{-kt}$ (where N

is the number of survivors at time t from an initial number No and k is a rate constant).

Further investigations on the loss of viability in bacterial populations reported deviations from the above-described log-linear relationship (Cerf, 1977), including curvilinear, concave (Fig 1.2E) or convex (Fig 1.2F), biphasic, with a "shoulder" (Fig 1.2D) or "tail" (Fig 1.2C), and triphasic deviations (Fig 1.2B), which are complicated by departures from the law of log-linear decline (Fig 1.2A) (McQuestin, 2006, Uyttendaele et al., 2001, Brown et al., 1997, Humpheson et al., 1998).

Concave curves (Fig 1.2 E) are generally considered to arise from heterogeneous populations as the cells exhibit different levels of tolerance to the inimical process (Lee and Gilbert, 1918). Biphasic, shoulders and tails, exhibit two slower distinct processes of log-linear decline in inactivation curves. Two hypotheses are proposed to account for shoulders. Firstly, each cell requires "multiple hits" before it is inactivated, so an initial lag prior to bacterial death occurs (Moats *et al.*, 1971). The second hypothesis is that in the cell population as a whole, cells may clump and protect a proportion of the population from the lethal agent (Tomlins, 1976).

Various explanations for tailing are proposed. Firstly, experimental artifacts due to faulty testing procedures or equipment may result in clumping of dead cells around those that are still viable, thus affording them protection (Cerf, 1977). Secondly, tailing could be a result of inherent variability within the population that is demonstrated by the appearance of a subpopulation of resistant cells (Cerf, 1977, Moats et al., 1971). Thirdly, tailing may be due to cells adapting to the stressful

environment and being able to survive the inimical process for longer (Cerf, 1977, Moats et al., 1971). Brown (2002) observed the triphasic inactivation curve depicted in Fig 1.2 B for *E. coli* populations when exposed to low pH. Phase 1 is considered as an initial and relatively rapid phase of inactivation, phase 2 is a slower rate of decline and phase 3, a final phase of inactivation, exhibits a rapid rate until viable cells cannot be detected by culture-based enumeration.

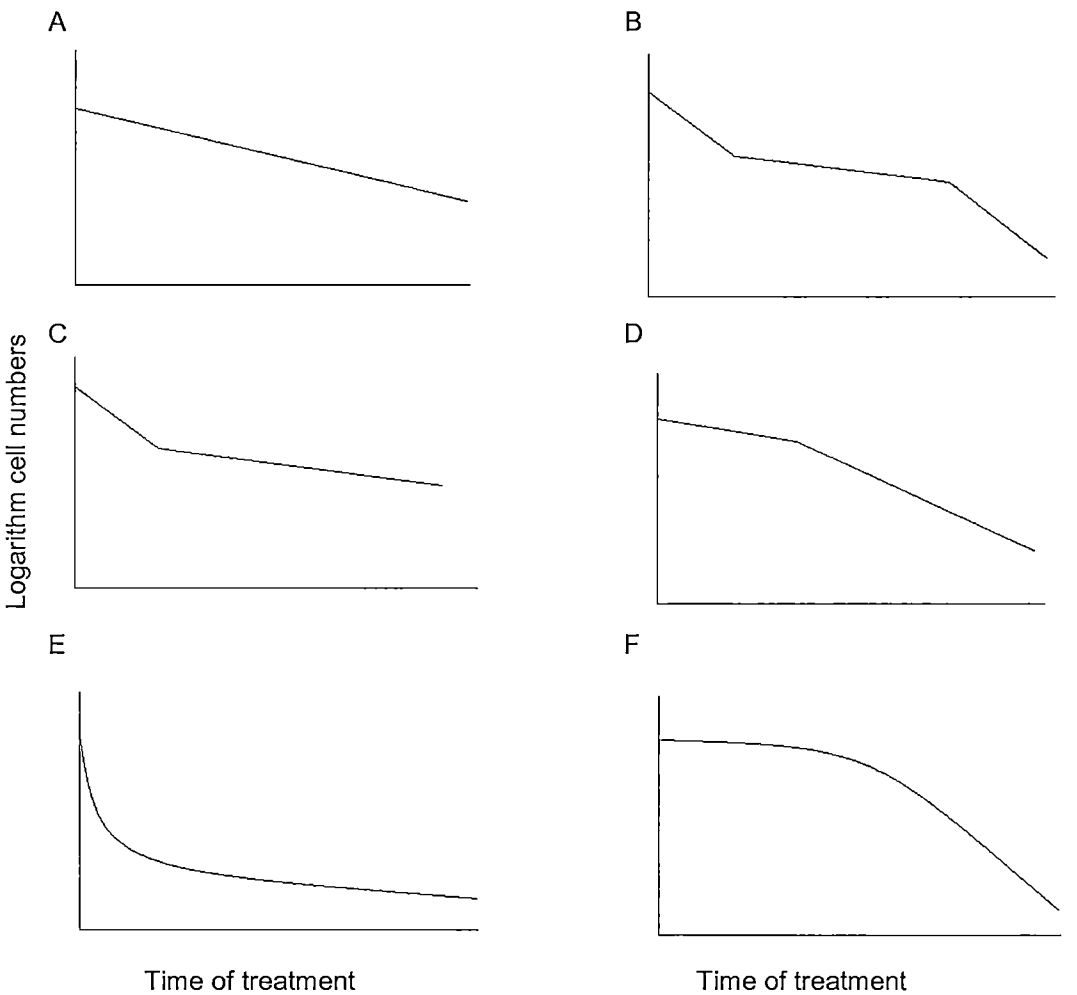


Fig 1.2 Common types of survival curves. A. Log-linear inactivation; B. Triphasic inactivation characterised by Phase 1, Phase 2 and Phase 3; C. Biphasic inactivation including "tailing" and containing phases analogous to Phase 1 and Phase 2; D. Biphasic inactivation with an initial "shoulder" and containing phases analogous to Phase 2 and Phase 3; E. Concave inactivation and F. Convex inactivation (McQuestin, 2006).

Humpheson et al (1998) used procedures that minimize experimental artifacts due to cell clumping, protective effects from dead cells and enumeration-related limitations and confirmed that the non-log-linear loss of viability of bacterial populations, which can be modified by experimental practices, is a real phenomenon, not an artifact.

1.5.2 Parameters Controlling Growth and Inactivation

Foods are complex ecosystems. A number of parameters may limit the growth and/or permit only the survival of bacteria in foods. Those properties inherent to the food itself are considered intrinsic factors: pH, water activity (a_w), nutrient content, oxidation-reduction potential (Eh), antimicrobial constituents and biological structures. Factors related to the storage environment are extrinsic parameters, such as temperature, relative humidity and gaseous atmosphere (Jay, 1992). For any environmental parameter, bacteria are able to grow only over a limited range. There is an upper and lower limit, where growth rate is minimal, or zero, and an optimal level, where growth rate is maximum. Bacteria become dormant and even inactivated, at the upper and, lower limit and/or beyond this permissable range for growth (Mellefont, 2000).

As multiple environmental parameter combinations are often applied to foods, the minimum and maximum level of each that permits microbial growth is often determined in experimental systems in which all other growth-controlling factors have been optimised. Among these parameters, the most influential factors on the growth and survival of pathogens are temperature, pH (acidity/alkalinity) and water

activity (saltiness/wetness) (Jay, 1992).

1.5.3 Temperature

The influence of temperature on microbial growth and physiology cannot be overemphasized. Microbial growth can occur over a temperature range from about –8°C up to 100°C at atmospheric pressure. No single organism is capable of growth over the whole of this range; bacteria are normally limited to a temperature span of around 35-40°C and moulds rather less, about 25-30°C (Ross and Nichols, 1999). Within most of the range, an increase in temperature increases the rate of the microbial response, whether growth/metabolism or inactivation. A typical bacterial growth rate curve is shown in Fig1.3. Bacterial growth rate increases with increasing temperature until the optimum temperature for growth is reached; it then decreases, falling to zero rapidly. All bacterial growth curves are of this general form, where temperature is the variable.

The growth rate response of bacteria to temperature conditions can be explained if bacteria are considered as chemical systems. In chemical systems, the effect of temperature is to accelerate reaction rates. The simplest explanation for this acceleration is called collision theory, where increased temperature raises the kinetic energy of reagent molecules, increasing the possibility of collision and reaction (Arrhenius, 1915). This explains the part of the temperature response curve where bacterial growth rate rises with increasing temperature.

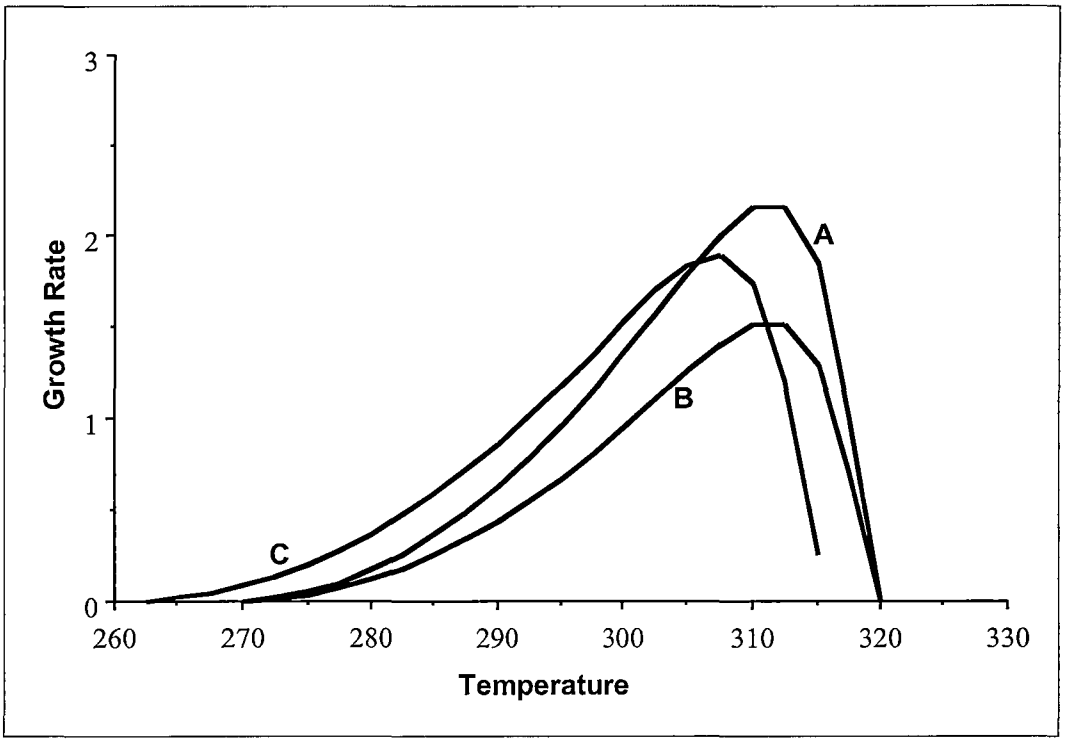


Fig 1.3 The general pattern of growth rate response to temperature, typical of bacteria. One organism growth rate response to temperature: under "optimum" conditions as curve A shows, while under "suboptimum" conditions as curve B indicates. The limits to growth and optima remain the same, but the growth rate is reduced at all temperatures. Curve C depicts the growth rate response to temperature of another organism that is, however, adapted to lower temperatures, (adapted from Olley et al.(1989)).

The thermodynamic relationship between bacterial growth or inactivation and temperature for chemical reaction is based on an Arrhenius's rate equation:

$k=Ae^{-E/RT}$

(where k is the specific reaction rate, A is a constant frequency factor with units of reciprocal time, E is the activation energy with units of calories mol^{-1}, R is the SI unit with a value of 8.314472(15) $J \cdot K^{-1} \cdot mol^{-1}$, and T is the absolute temperature (Rose, 1983). Some theories of microbial growth rate response assume that growth and inactivation to temperature is governed by a single rate-limiting enzyme catalysed

reaction (McMeekin and Ross, 1993).

Fig 1.4 shows an Arrhenius plot and the response over the mid-range of temperature is referred to as the "normal physiological range" and describes where the response is linear on Arrhenius coordinates. At and beyond the maximum and minimum temperatures for growth, the asymptote deviates from Arrhenius kinetics and approaches vertical. The slope of the linear region and cardinal temperatures for growth vary widely among species, however, the general form of the Arrhenius plot of growth rates can be applied to all bacteria (Ingraham and Marr, 1996).

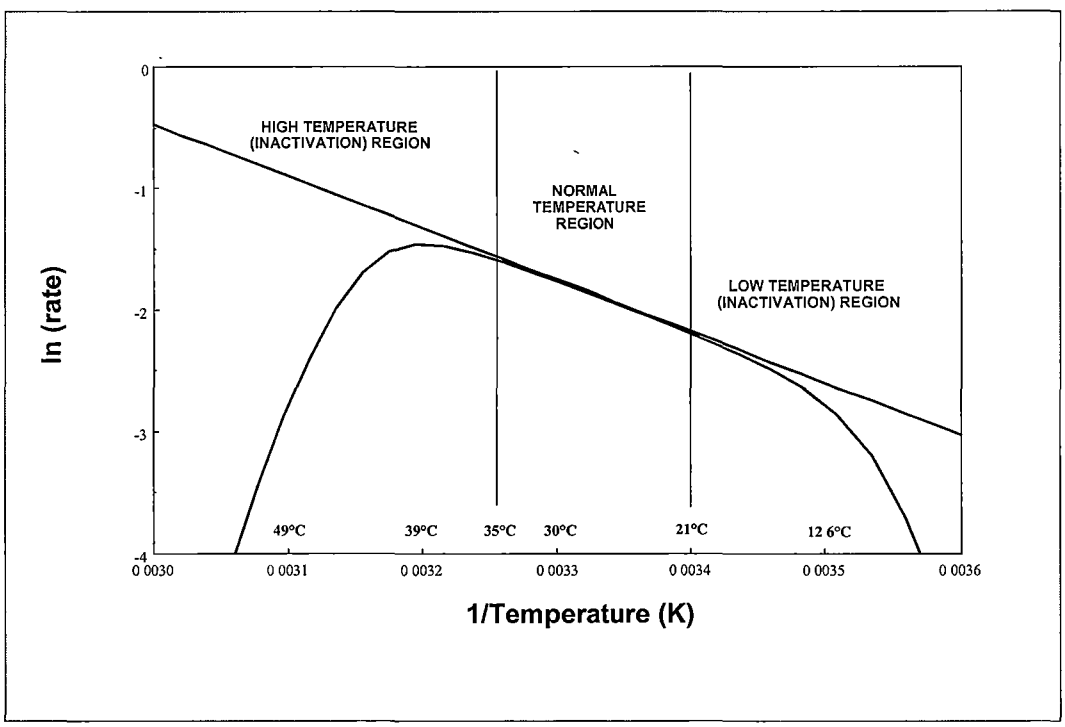


Fig 1.4 The rate of growth of *E. coli* as a function of temperature and is presented as an Arrhenius plot which graphs the logarithm of rate of growth as a function of the reciprocal of absolute temperature (adapted from Ratkowsky et al.(2005).

The reduction in growth rate above the optimum temperature can be explained by

the fact that heat causes break in single strands of deoxyribonucleic acid (DNA), increases the non-selective permeability of the cytoplasmic membrane and denatures proteins therefore damaging cellular enzymes (Sizer, 1943, Gould, 1989b). Bacteria use enzymes to catalyse their chemical reactions; however, the function of these enzymes is dependent on their being in an active conformation (native state) or inactive conformations (denatured state). Heat denaturation times are much shorter than cold denaturation, explaining the sharp reduction in growth rate at the high temperature growth limit (Sharpe and DeMichele, 1977)

1.5.3.1 *E. coli* and Temperature

Like most bacteria, *E. coli* grows over a temperature range of approximately 40°C, although the exact optimum, minimum and maximum temperatures vary between strains. The minimum temperature for growth of *E. coli* is approximately 8°C (Ingraham and Marr, 1996), whereas the optimum and maximum temperatures are around 40 and 46-48°C respectively (Salter et al., 1998). Pathogenic strains of *E. coli* generally display similar growth characteristics to non-pathogenic *E. coli* in response to temperature (Salter et al., 1998).

1.5.3.2 *L. monocytogenes* and Temperature

L. monocytogenes growth occurs over a range of 0 to 45°C, and the optimal growth temperature is between 30 and 37°C (Junttila *et al.*, 1988). Inactivation occurs when exposed to temperatures above 50°C, but the D value at 50°C can be in the order of hours. On the other hand, the survival of *L. monocytogenes* is enhanced with lower temperatures (Junttila *et al.*, 1988). Its fastest doubling time is in the range of 35-40

minutes, and occurs at 37°C, where pH is neutral, and in rich medium that contains sufficient nutrients and has an a_w in the range 0.990 to 0.995 (1±0.5% NaCl) (Seeliger and Jones, 1986).

1.5.4 Water Activity

Water is the solvent of life. Water availability is generally expressed in physical terms such as water activity or water potential. Water activity, abbreviated a_w, is expressed as a ratio of the vapor pressure of the air over a substance or solution divided by the vapor pressure at the same temperature of pure water. Thus values of a_w vary between 0 and 1. Pure water has a a_w of 1.00, a 15% (weight per volume) NaCl solution has a a_w of approximately 0.90 and a saturated NaCl solution has a a_w of 0.75 (Chirife and Resnik, 1984).

a_w is considered the most important environmental influence on microbial growth and survival in dry foods. Traditional techniques for the preservation of perishable food, such as salting and drying, manipulate the a_w of the food matrix, despite that the mode of action of this lethal agent has not be fully elucidated. Changes in a_w can be achieved by the addition of humectants such as sugar or salt, as well as by drying or freezing to either retard microbial growth or induce microbial death. a_w in a food matrix is not the total water content of the food itself, because water-soluble salts, proteins and carbohydrates bind a proportion of that water (Troller and Christian, 1978, Brock and Madigan, 1991).

Although it varies among species, the response curve of microorganisms to a_w, is similar to that for temperature. Below and above the optimum a_w for growth, growth

rate declines approximately linearly with decreasing a_w, but for the range above the optimum with a steeper tangent shows in Fig 1.5. It is assumed that most bacteria will be unable to grow below an a_w value of 0.8 (Beales, 2004).

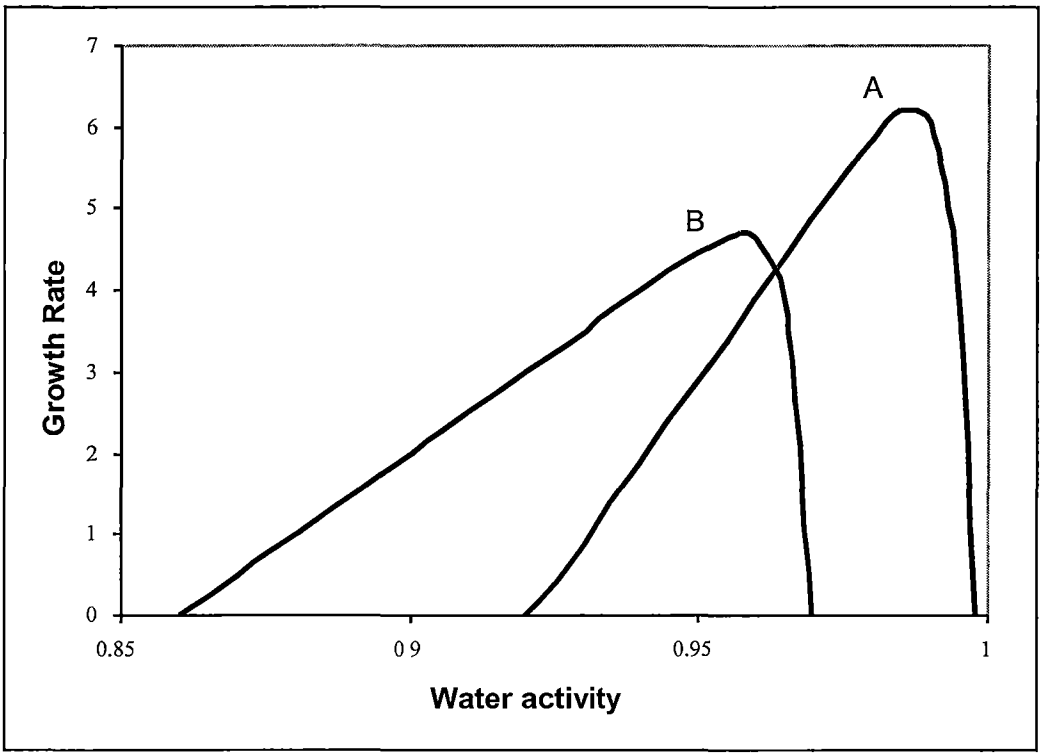


Fig 1.5 The general pattern of growth rate response to water activity, typical of bacteria, showing two different organisms A and B on the effect of water activity (adapted from http://www.bact.wisc.edu/Microtextbook/index.php?name=Sections&req=viewarticle&artid=12&allpages=1&theme=Printer).

1.5.4.1 Physiological Analysis

The bacterial cell membrane plays an important role in separating the cytoplasm and the surrounding environment. It is the main barrier allowing free passage of water and selective exchange of solutes. There are two parts of the cell membrane-lipids and proteins, and the major lipid components of the bacterial cell membrane

are phospholipids, which maintain normal function of the bacteria cell membrane, so water can move across the lipid bilayer by osmosis (Csonka and Epstein, 1996).

An increase in external osmolality is referred to as an osmotic "upshock" and occurs when water diffuses out of the cell. Under extreme conditions this results in plasmolysis where the cell membrane shrinks away from the cell wall. An osmotic "downshock" refers to the influx of water into the cytoplasm, which can lead to rupturing of the membrane. The term osmotic stress is usually used to refer to the stress of adapting to an environment of high osmolality, high salinity or low a_w (Csonka and Epstein, 1996). Osmotic stress also leads to changes in membrane lipid composition, which is part of the osmosensing mechanism, as an increase in the proportion of anionic membrane lipids (diphosphatedylglycerol and phosphatidylglycerol) and a decrease in the proportion of zwitterionic counterparts (phosphatidylglycerol), which helps restore the lipid bilayer phase of the membrane (Beales, 2004).

Bacteria adjust their cytoplasmic water activity using two strategies: the salt-in-cytoplasm type where prokaryotes accumulate potassium chloride to high intracellular concentrations, and the organic-osmolyte-in-cytoplasm type where most bacteria use organic solutes accumulated in the cytoplasm from the environment for osmoadptation (Trüper and Galinski, 1989). Unlike the salt-in-cytoplasm strategy, the latter is a more flexible mechanism because the organic solutes balance osmotic pressure but do not inhibit the functioning of salt-sensitive enzymes. Since they are compatible with enzymes, the organic osmolytes are often called compatible solutes (Brown, 1976). Both the strategy and compatible solutes

used are common to all groups of organisms (Yancey *et al.*, 1982). Therefore, compatible solutes have fundamental properties in osmoadaptation of all biological systems.

Compatible solutes have low molecular weights and polar function, which make their molecules highly soluble and facilitates their accumulation to high intracellular concentrations. They are usually neutral or zwitterionic molecules, uncharged at normal cytoplasmic pH values, have little effect on the activity of cytoplasmic enzymes and may protect them from denaturation by salts (Bremer and Kramer, 2000). Compatible solutes are usually end-product metabolites, rather than intermediaries in bio-synthetic pathways and, therefore, tend to be stable once formed (Duche et al., 2002a, Beales, 2004). Trüper and Galinski (1989) identified four classes of compounds as potential compatible solutes: sugars, polyols, amino acids and betaines. Csonka and Epstein (1996) then compiled a list of all compounds that have been implicated as compatible solutes in osmoregulation: sugar polyol derivatives (glucosyl glycerol); zwitterionic trimethyl ammonium and dimethylsulfonium compounds (betaines, thetines); natural amino acids (proline, glutamine); glutamine amide derivatives (Na-carbamolylglutamine amide); N-acetylated diamino acids (Nδ-acetylornithine); ectoines (ectoine, β-hydroxyectoine). Most of these compatible solutes are synthesisied by halophilic and/or halotolerant bacteria, but many are accumulated by non-halophiles in response to osmotic stress. Non-halophiles also use potassium glutamate and sugars such as trehalose as compatible solutes.

Among the spectrum of compatible solutes, glycine betaine, ectoine, proline and

trehalose are probably the most widely used in the microbial world (Bremer and Kramer, 2000, Smith, 1996). Compatible solutes can be either transported into the cell or synthesized *de novo*. The availability of compatible solutes in the environment determines whether synthesis or uptake will predominate in their cellular accumulation, and whether their intracellular concentration is sensitively adjusted to the degree of osmotic stress. This process is controlled by an interplay between mechanisms that regulate either gene transcription or activity of the encoded enzymes and transporters used for compatible solutes accumulation (Bremer and Kramer, 2000, Bayles and Wilkinson, 2000).

Generally, the internal osmotic pressure in bacterial cells is greater than that of the surrounding environment and so pressure is exerted by the cytoplasmic membrane on the cell wall; this is called turgor pressure or turgor (Bremer and Kramer, 2000). Therefore, to survive fluctuations in the water content of their habitat, bacterial cells must maintain turgor within physiologically acceptable boundaries. Upon osmotic downshock, the excess compatible solutes are rapidly expelled in a controlled manner through the stretch-activated channels to allow the cell to control water content and turgor and thus sustain growth under unfavorable conditions. However, the upshock response is totally opposite (Bremer and Kramer, 2000). Bacteria maintain a positive turgor pressure in low water activity conditions using a rapid primary response immediately after an osmotice up-shift, namely, efflux of water by diffusion across the lipid bilayer and through aquaporins (Bremer and Kramer, 2000). Among many physical and structural changes in the cell that presumably trigger the osmosensing process, the primary response is massive uptake of K^+ ions from the environment. This occurs immediately after the osmotic shift and is under

the control of potassium transport systems. During the response, potassium ions are accumulated and glutamate is synthesized, because high cyoplasmic K^+ concentrations affect the conformation of macromolecules, which has a negative affect on protein function and DNA-protein interactions. This response is an inadequate strategy for coping with longer-term osmotic stress. So, within a few minutes, the accumulation of compatible solutes to discharge large amounts of the initial acquired K^+ through efflux systems is essential in the second response, after the initial increase in cellular K^+ concentrations (Duche et al., 2002b, Smith, 1996, Bremer and Kramer, 2000).

1.5.4.2 Low a_w stress and *E. coli*

E. coli is a non-halophilic organism (Ingraham and Marr, 1996). Like other bacteria, *E. coli* maintains an internal a_w at a lower level than that outside the cell, i.e. a positive turgor, establishing a tendency for water to flow into the cell. Maintenance of turgor is essential for bacterial growth, since it is vital for the elongation of cells during division (Koch, 1982). Osmotic stress decreases or damages turgor, which will inhibit growth. DNA replication is also inhibited at low a_w (Meury, 1988). The minimum a_w permitting growth of *E. coli* is 0.95, or about 8% sodium chloride (Gould et al., 1977, Salter et al., 2000, Troller and Christian, 1978). This minimum for growth is influenced by pH and temperature, with effects most marked at low temperatures and low pH (Desmarchelier and Grau, 1997).

The passage of water across the bacterial cell membrane is passive compared to the active influx and efflux of various solutes from cells in response to osmotic stress. It will effectively decrease the a_w of the cytoplasm, reduce further water efflux and

contribute to the loss of turgor (Bremer and Kramer, 2000). However, loss in turgor *per se* does not appear to result in a loss in viability of bacterial cells (Gould, 1989a), which means the processes of bacterial growth inhibition and inactivation are distinct in response to some stresses, probably due to separate mechanisms that are involved. The mechanism of bacterial inactivation due to low a_w is still unclear, although there has been 30 years of research since the monograph of Troller and Christian (1978). There are a number of questions that remain to be answered: i) Since low a_w conditions can damage cellular sites, i.e. cell wall and the cell membrane (Mossel and Netten, 1984), whether such injury is merely damage, or is to some extent lethal and actually kills the cell. ii) There is an increasing suggestion that the damage caused is due to the changes to the structure of water in the bacteria cell, but how low water activity inhibits cells growth needed to be elucidated and iii) How those changes affect microbial molecules in the cell. Nevertheless, osmoregulation, a specific physiological response initiated by *E. coli* as an aspect of the low a_w effect on bacteria, has been better defined as described above.

1.5.4.3 Low a_w stress and *L. monocytogenes*

Although it is a Gram-positive organism and is more salt tolerant than *E. coli*, *L. monocytogenes* shares common responses to low water activity. Its optimal growth a_w is $\geq$0.97 (Seeliger and Jones, 1986), and minimum a_w for growth is 0.92 with NaCl ($\approx$ 11.5% NaCl) (Farber *et al.*, 1992). In addition, it can survive in 20% NaCl for 8 weeks at 4°C or 10.5% NaCl for 15 days at 37°C (Seeliger and Jones, 1986), i.e. lowering the temperature can increase the survival in high-salt conditions.

Similar to *E. coli*, the cytoplasmic accumulation of K^+ and its counterion, glutamate,

was observed in *L. monocytogenes* following a sudden increase in the osmolarity (NaCl, 7.5% w/v) (Patchett et al., 1992). In contrast, *L. monocytogenes* possesses two K^+ transporters, a high-affinity system, KdpABC, which exhibits significant similarity to Kdp of *E. coli*, and a low-affinity system, lmo0993, which resembles both Trk system of *E. coli* and KtrII system of *Enterococcus hirae* (Sleator et al., 2003a). However, this primary response has received considerably less attention than the secondary response, which has been thoroughly investigated at both the gene and protein levels, with most focus on the trimethyl ammonium compound glycine betaine (N, N, N-trimethylglycine), triethyl amino acid γ-N-trimethyl aminobutyrate (L-carnitine) and proline (Patchett et al., 1992, Sleator et al., 2003b, Ko and Smith, 1999). In addition, certain peptides, specifically the proline-containing di- and tripeptides prolyl-hydroxy-proline (PHP) and prolyl-glycyl-glycine (PGG), also function as effective osmoprotectants, causing increase in the growth rate at 4% NaCl (Amezaga *et al.*, 1995). Osmoprotectants for *L.monocytogenes* are approximately ranked in the order as follows: glycine betaine> proline betaine> carnitine ~ acetyl carnitine ~ γ-butyrobetaine > 3-dimethylsulphoniopropionate (Bayles and Wilkinson, 2000). In addition, the absolute amount of each compatible solute accumulated is dependent on the temperature, the compatible solute itself, and the growth phase of the culture. While the initial choice is betaine, carnitine appears to play an increasingly important role in osmoregulation as the culture ages (Smith, 1996).

Little information is available concerning other mechanisms that *L.monocytogenes* uses to cope with salt stress, especially when compatible solutes are not available in the environment. A general stress protein encoded by *ctc*, was shown to be involved

in the resistance of *L.monocytogens* to high osmolarity in the absence of osmoprotectants such as betaine and carnitine in the medium (Gardan *et al.*, 2003). Rel, encoded by *relA*, encodes guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) synthetases, collectively designated (p)ppGpp, and it is also essential for full osmotolerance. Its mechanism is different from that for the compatible solutes (Okada *et al.*, 2002). HtrA, identified in *L. moncytogenes* as a serine protease, plays a role in degrading improperly folded proteins that accumulate under stress conditions of high osmolarity, heat, or hydrogen peroxide stress (Wonderling *et al.*, 2004). Two genes, *clpC* and *clpP*, encoding a ClpC ATPase, a general stress protein involved in intracellular growth and *in vivo* survival in host tissues, and a ClpP serine protesase, respectively, have been identified. Inactivation of these genes conferred a general stress sensitivity phenotype, including sensitivity to salt stress, to the corresponding mutant (Rouquette *et al.*, 1996).

1.5.5 pH

Acidity or alkalinity of a solution is expressed by its pH value on a scale in which neutrality is pH 7, where most microorganisms grow best. pH values less than 7 are acidic and greater than 7 are alkaline. It is important to remember that pH is a logarithmic function; a change of one pH unit represents a ten-fold change in hydrogen ion concentration (Jay, 1992).

Microorganisms commonly live in widely fluctuating pH environments. Although it varies among species, the optimal pH for bacterial growth usually occurs in the middle of the pH range around 7.0 (6.6-7.5), whereas a few grow below 4.0 (Gould,

1989b). The region of fastest growth occurs over a range of pH surrounding the optimum, usually 1 to 2 pH units, as Fig 1.6 shows. Growth rate rapidly declines to zero near the maxima and minima, the values of which may vary between microorganisms, and these values may be affected according to the type of acidulant and alkali in their environment. In addition, other environmental stresses will raise the minimum pH that permits growth. For example, decreasing a_w values will narrow the pH range over which microorganisms can grow (Mossel *et al.*, 1991). And as little as 0.2 pH units change can be sufficient to elicit significant change in cell physiology (Booth et al., 1999).

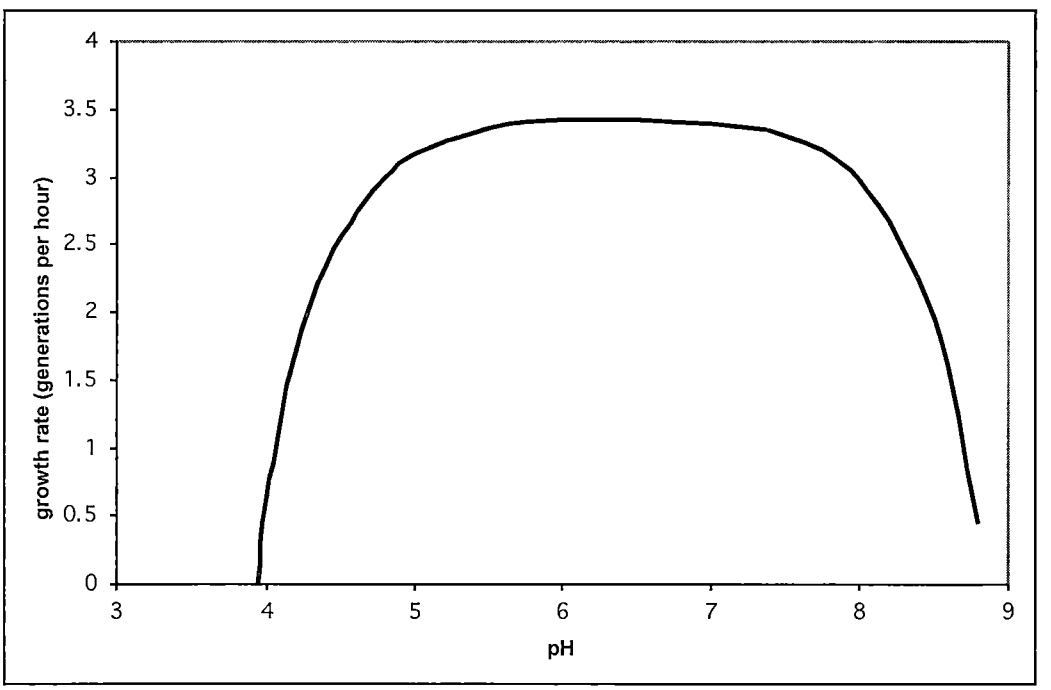


Fig 1.6 The general growth rate response of microorganisms to pH. The organism shown is neutralophile, having an optimal growth rate at near-neutral pH. As the pH increases or declines more than about 1 pH unit from neutrality, the decline in growth rate becomes apparent (adapted from Presser (2001)

Low pH is one of the most common stresses encountered by foodborne

microorganisms in unprocessed foods, during fermentation and using organic acids as a means of food preservation (Bearson et al., 1997, Archer, 1996, Booth and Kroll, 1989). Low pH equates to a high concentration of hydrogen ions and is often referred to as an acidic pH. Increasingly acidic conditions reduce the potential for microbial growth and ultimately bring about microbial inactivation (Bearson *et al.*, 1997).

Before describing the prokaryotic responses to acid stress, acid stress itself must be defined. Acid stress can be described as the combined biological effect of low pH and organic (weak) acids present in the environment. Organic acids, including volatile fatty acids (VFAs) like butyrate, propionate and acetate produced as a result of fermentation, are generally more effective than inorganic acids at inhibiting bacterial growth. This is because, unlike inorganic acids, organic acids in their uncharged, protonated forms can diffuse across the cell membrane and dissociate inside the cell, releasing hydrogen ions, lowering internal pH (pH_i) in the process (Corlett and Brown, 1980). The lower the external pH (pH_o), the more undissociated organic acid will be available (based upon pK_a values) to cross the membrane and affect pH_i. This means that it takes less organic acid to kill a cell at pH_o3.5 than is needed at pH_o 4.4. Intracellular accumulation of organic acids is also thought to have harmful effects on the cell beyond that of acidifying pH_i. It is important to remain cognizant of the relationship between pH and organic acid concentration when considering cellular acid survival strategies (Corlett and Brown, 1980). At the same pH, the antimicrobial activity is acetic acid> lactic acid> citric acid> malic acid > HCl and is correlated with the pKa of the organic acid (Sorrells et al., 1989, Vasseur et al., 1999). Moreover, the acid tolerance is strongly dependent on the

growth temperature as cells became less resistant to acid as the temperature decreases (Koutsoumanis *et al.*, 2003).

1.5.5.1 Physiological Analysis

Bacteria cannot tolerate large changes in the pH_i, as hydrogen ions have a destabilizing effect on biological macromolecules (Foster, 2000, Booth and Kroll, 1989). Therefore, when a pH stress is encountered, the cell not only has denatured enzymes present on the cell surface, but the cell also pumps hydrogen ions across its membrane to maintain a relatively constant pH_i, which provides an explanation for the plateau portion of the pH-response curve as Fig 1.6 shows. The points where growth rate drops rapidly, at the acidic and alkaline limits for growth, is probably due to the breakdown of homeostatic mechanisms, and therefore loss of control over intracellular pH_i and then, markedly reduced growth rates (Presser *et al.*, 1997). This is because pH must be maintained within critical values beyond which intracellular proteins become irreversibly denatured (Montville, 1997). In addition, the pH of the external environment has to be changed by several pH units before pH_i is affected.

The response process is achieved by three progressively more stringent mechanisms: a homeostatic response, the acid tolerance response and synthesis of acid shock proteins (Montville, 1997). These three mechanisms are incompletely understood.

The ability to maintain pH_i within a narrow range of values despite variations in the pH_o is termed pH homeostasis, which depends on a combination of passive and active mechanisms. Passive components are the lipid composition of the membrane

and the buffering capacity of the cytoplasm, while active homeostasis involves control over the movement of K^+, Na^+ and H^+, and alterations in the membrane permeability for any of these cations may cause perturbation of homeostasis (Brown, 2002). A good example is *Salmonella.* At $pH_o > 6.0$, *Salmonella* cells adjust their pH_i through the homeostatic response, which maintains pH_i by allosterically modulating the activity of proton pumps, antiports, and symports to increase the rate at which protons are expelled from the cytoplasm. The homeostatic mechanism is constitutive and functions in the presence of protein synthesis inhibitors. These are reviewed by Booth (1985).

A drop in pH_i coincides with a net influx of protons suggesting that membrane transport processes are the main contributors to the process of pH homeostasis. At low pH, a decrease in the ratio of unsaturated to saturated fatty acids coupled with an increase in the amount of cyclopropane fatty acids is observed. The result of decrease in the fluidity of the membrane prevents the movement of protons across the membrane, therefore reducing the pH_i (Booth, 1999, Beales, 2004). In addition, buffering due to synthesis of intracellular metabolites also prevents pH_i disruption (Foster, 2000). Furthermore, bacteria commonly modify pH_o through metabolic activities and extrusion of compounds that can accept H^+ ions , however, this requires a continuous access to suitable substrates in sufficient quantity (Dilworth *et al.*, 1999).

After exposure to mild acidic conditions (e.g. pH_o of 5.5 to 6.0), bacteria acquire improved ability to survive lethal acid concentrations. This inducible mechanism is termed the acid tolerance response (ATR) (Foster and Hall, 1991). This mechanism

is sensitive to protein synthesis inhibitors; at least 18 ATR-induced proteins have been identified. ATR appears to involve the membrane-bound ATPase proton pump and maintains $pH_i > 5.0$ at pH_o values as low as 4.0. The loss of ATPase activity caused by gene disruption mutations or metabolic inhibitors abolishes the ATR, but not the pH homeostatic mechanism described above. The ATR may confer cross-protection to other environmental stressors. The exposure of *S. typhimurium* cells to pH 5.8 for a few doubling induces 12 proteins, represses 6 proteins, and renders the cells less sensitive to salt and heat (Leyer and Johnson, 1993). Acid adaptation also occurs in *E.coli* O157: H7 (Leyer *et al.*, 1995).

The synthesis of acid shock proteins is the third way that cells regulate pH_i. The synthesis of these proteins is triggered by pH_o from 3.0 to 5.0. They constitute a set of transacting regulatory proteins distinct from the ATR proteins. They may be similar to cold shock proteins which help confer acid resistance in *L. monocytogenes* (Foster *et al.*, 1994).

1.5.5.2 Low pH stress and *E. coli*

Although *E. coli* must pass through the acidic gastric barrier to cause gastrointestinal disease; it is a neutralophile with regard to pH. It is generally accepted that in laboratory media, *E. coli* grow in the range of pH 4.4 to pH 10 (Desmarchelier and Grau, 1997). However, Presser et al. (1997) have demonstrated a pH limit in the range 3.9 to 4.0. As with other neutralophiles, growth rates for *E. coli* are maximal between pH 6.0 and 8.0, and are slower at half a pH unit or so beyond these limits (Ingraham and Marr, 1996). The minimum pH for growth of *E. coli* is dependent on the type of organic acid present and its concentration.

Homeostasis breaks down only at the more extreme conditions.

Two principal systems are recognized as potential generators of pH gradients. These systems are potassium-proton antiporters and sodium-proton anitiporters. As a simplified overview, shifts to acidic environments are handled by K^+/proton antiporters, resulting in alkalinizaiton of cytoplasm (Booth and Kroll, 1989).

1.5.5.3 Low pH stress and *L. monocytogenes*

A neutrophile, *L. monocytogenes* grows best in the pH range 6-8 and maintains its pH_i values at a constant level around pH 7.6 (Foster, 2000). In general, *L.monocytogenes* grows over the pH range of 4.2 – 4.3 to around 9.6 (Seeliger and Jones, 1986). Intracytoplasmic pH measurements showed that different strains of *L. monocytogenes* have different pH_i value at the same pH_o. This suggests that different strains possess different cell membrane characteristics leading to different pathways of H^+ influx and efflux across the cell membrane for a given pH_o (Phan-Thanh *et al.*, 2000). In addition, at the onset of the stationary phase, *L.monocytogenes* displays the higher level of acid tolerance than exponential phase, and when entering the stationary phase, *L. monocytogenes* possesses generalized stress resistance resulting in a pH-independent acid tolerance response (ATR) and acid resistance (AR), which is regulated by an alternative sigma factor, σ^B (Davis et al., 1996, Samelis et al., 2003).

It is evident that the fate of microorganisms is affected by a complex interplay of environmental parameters. Consequently there are opportunities for control of microorganisms in foods by manipulation of these parameters. However, for non-

thermal inactivation there are many areas of uncertainty: What is the molecular mechanism of osmo- and/or pH-sensing and which physiological and physical parameters are actually sensed by cells subjected to sudden osmotic and/or acidic increases or prolonged survival or inactivation in high osmolality and/or acidity environment, and what is the physiological or molecular mechanism of cell inactivation or death due to osmotic and/or acidic stress?

1.6 OBJECTIVES

Within the above context the aim of this thesis is to expand understanding of the kinetics, methods and mechanisms whereby temperature, low a_w and pH effects on the inactivation of *E. coli* and *L. monocytogenes*, specifically by:

i) Reviewing the literature concerned with the classification, pathogenicity and susceptible populations of vegetative bacteria, *E. coli* and *L. monocytogenes*, respectively, with their response to temperature, low a_w and pH, and the physiological and molecular basis of those responses [Chapter 1]

ii) Based on previous studies (Ross and Shadbolt, 2001, McQuestin, 2006) of the importance of temperature on governing the inactivation rate of *E. coli* in fermented meat products, investigating the role of temperature in the rate of inactivation of vegetative bacteria, not only *E. coli* but also *L. monocytogenes*, once sufficient hurdles are in place to prevent growth [Chapter 2]

iii) Investigating the utility of ATP bioluminescence as a sensitive and rapid method for assessing inactivation of microbial populations in broth culture based on

physiological level of cell response to inimical stresses [Chapter 3], and as an alternative to labour-intensive, viable count, method.

iv) Undertaking studies to elucidate mechanisms that inactivate food borne pathogens in inimical food environments, specifically using QPCR method developed to indicate cell viability, and to explore possible physiological processes of non-thermal inactivation of a cell [Chapter 4]

v) Further exploring the mechanisms of non-thermal inactivation, using time-course DNA microarray investigations of low pH and a_w stresses on *L. monocytogenes* ScottA [Chapter 5]

From a practical point of view, the project attempts to extend the framework available for the development of strategies designed to ensure microbiologically safe foods using traditional, non-thermal, food preservation methods.

CHAPTER 2

AN INVESTIGATION OF THE ROLE OF TEMPERATURE IN THE INACTIVATION RATE OF VEGETATIVE BACTERIA

2.1 ABSTRACT

Experiments were undertaken to determine the relative influence of temperature on the survival of two species of food-borne bacteria under growth-preventing pH and water activity conditions. Inactivation rates for four strains of *E. coli* and three strains of *L. monocytogenes* were determined in a complex nutrient media adjusted to a_w 0.900 and pH 3.50 and at nine temperatures in the range 5 to 45°C at 5°C intervals. Sixty-three inactivation rates were generated, plotted on Arrhenius co-ordinates and lines of best-fit determined by simple linear regression to enable the comparison of the effects of temperature on non-thermal inactivation of the two, physiologically distinct species. The inactivation rates and kinetics of *E. coli* and *L. monocytogenes* were very similar at each temperature apart from 45°C, and the inactivation rate responses of both were comparable to those previously and independently reported for a variety of *E. coli* strains under a wide range of growth-preventing pH and water activity conditions. Taken together with previous reports the results support the hypothesis that temperature is the dominant factor governing inactivation of vegetative bacteria when they are prevented from growth by other environmental factors.

2.2 INTRODUCTION

The production of microbiologically safe foods relies upon the application of stressful conditions that inhibit or prevent the growth and survival of contaminating pathogens. The preservation of almost all foods relies on multiple techniques to provide a series of 'hurdles' to microbial viability, either applied empirically or using the concept of hurdle technology (Leistner, 2000). These hurdles may include temperature (heating and refrigeration), acidity, water activity (a_w), redox potential, irradiation, antimicrobial compounds and others. Of the numerous hurdles used in food preservation, the most influential on the survival of microorganisms, and the most common, are temperature, pH and a_w (Adams and Moss, 2000).

E. coli is an extensively studied bacterium, both as a model Gram-negative microorganism and, more recently, as a life-threatening food-borne pathogen. Outbreaks of infection have been associated with a variety of food products including ground beef (Riley et al., 1983; Willshaw et al., 1994), pasteurised and raw milk (Upton and Coia, 1994; Keene et al., 1997; Goh et al., 2002), cheese (Honish et al., 2005), apple cider (Besser et al., 1993; Cody et al., 1999) and salad produce (Hilborn et al., 1999; Michino et al., 1999). Furthermore, pathogenic strains have been implicated in serious food-borne disease outbreaks involving fermented meats products, such as salami and mettwurst (CDC, 1995; Tilden et al., 1996; Williams et al., 2000; MacDonald et al., 2004). Although fermented meat products are typically not heat-treated prior to consumption, they had previously been considered safe from pathogenic *E. coli* due to their reduced pH and a_w.

To better understand the potential for survival of *E. coli* in fermented meats, Ross and Shadbolt (2001) undertook a "meta-analysis" of published data for *E. coli* under

a wide range of pH and a_w conditions that prevented growth, but at temperatures that would otherwise permit growth. Those investigators identified the usefulness of plotting microbial inactivation rates in growth-preventing environments as an Arrhenius plot (ln(inactivation rate)-vs-1/absolute temperature). From that diversity of independent studies, at various combinations of a_w, pH, lactic acid concentration and oxygen availability, it was shown by simple linear regression fitted to *E. coli* inactivation data on an Arrhenius plot, that temperature explained 64% of the variance in the observed *ln*(inactivation rate) data for all studies combined.

Ross *et al* (2004) extended that collation of published and unpublished sources of non-thermal inactivation rates of *E. coli*. In addition, they generated an extensive data set of *E. coli* inactivation rates in fermented meats, and a broth model designed to mimic the physico-chemical conditions in fermented meats. After calculation of the inactivation rates, the data were plotted on the Arrehnius co-ordinates, and straight lines were fitted to each of the data sets (Fig 2.1). Despite that the two data sets were independently derived and not from co-ordinated experiments, the lines of best fit were virtually indistinguishable. This reinforced the observation that the overall inactivation rates of *E. coli* in fermented meats and analogous systems in which pH, lactic acid and a_w alone or in combination prevented growth, while variable within individual trials are, on average, consistent. Thus, even when temperature *per se* is not stressful to the cells, i.e. when *E. coli* inactivation is due to the other environmental factors, temperature still has the greatest effect on the rate of inactivation of *E. coli*.

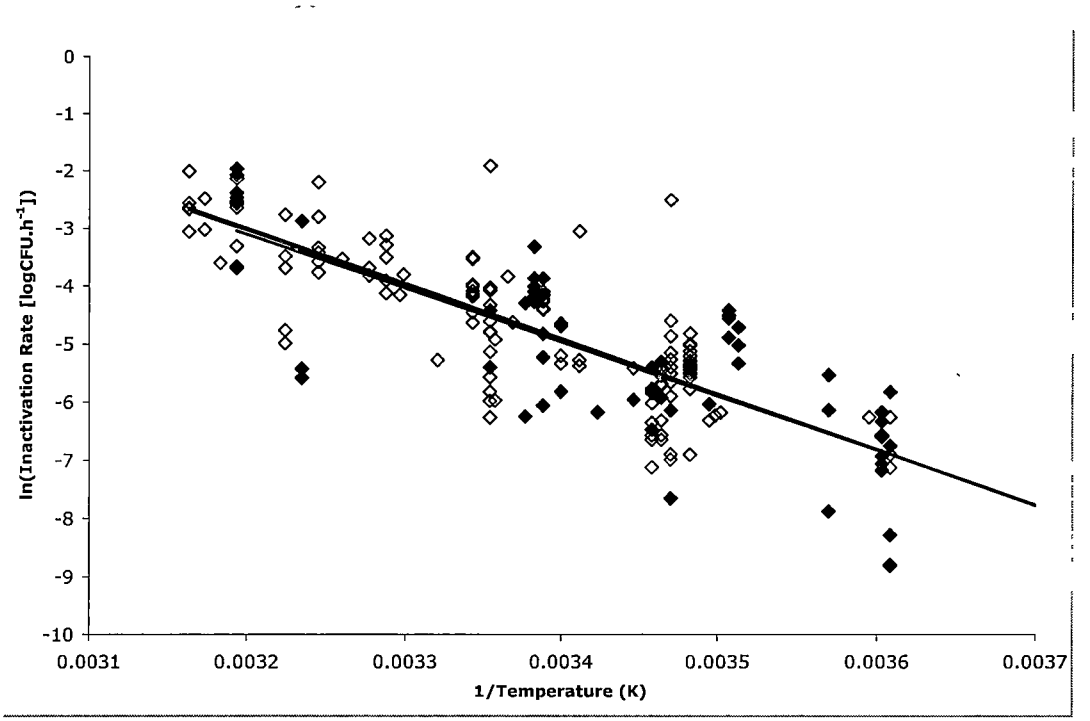


Fig 2.1 Comparison of 'lines of best fit' through "old" (open diamonds) data for *E. coli* inactivation in UCFM data (Ross and Shadbolt, 2001) and "new" (closed diamonds) data sets collated by Ross et al., (2004)

In this chapter experiments were designed and undertaken to assess the generality of the above observations by testing the hypothesis that temperature is not only the greatest influence on inactivation of *E. coli*, a Gram-negative organism, but that the observation is true of other vegetative bacteria.

L. monocytogenes has assumed importance as another major agent of food-borne disease during the past two decades, and is associated with a range of fresh food product of animal or plant origin (Khelef et al., 2006). Being a Gram-positive organism its physiology differs from that of *E. coli*, as does its ability to grow under different stress conditions. For example, *L. monocytogenes* is better adapted for

growth in slightly colder environments than *E. coli* and also withstands lower a_w. A comparison of the temperature, pH and a_w growth limits of *E. coli* and *L. monocytogenes* is shown in Table 2.1. Due to these differences, *L. monocytogenes* was chosen as a second bacterium to study the effect of temperature on non-thermal inactivation.

Table 2.1 Growth limits of *E. coli* and *L. monocytogenes*, due to three main environmental factors, water activity (a_w), temperature and pH.

Bacteria / Factors	*E. coli*		*L. monocytogenes*	
	Minimum	Maximum	Minimum	Maximum
Water Activity (a_w)	~0.95[a]	>0.998[d]	~0.92[e]	>0.998[e]
Temperature (°C)	~7.5[b]	~49[d]	0[f]	45[f]
pH	~3.9[c]	10[c]	~4.2[g]	~9 6[g]

a:(Gould *et al* , 1977); b·(Buchanan and Bagi, 1994); c:(Desmarchelier and Grau, 1997); d:(Salter *et al.*, 2000), e.(Farber *et al* , 1992); f·(Junttila et al , 1988a); g·(Seeliger and Jones, 1986).

2.3 MATERIALS AND METHODS

2.3.1 Bacterial Strains, Media, Reagents, Solutions and Equipment

Details of bacterial strains, bacteriological media, chemical reagents, solutions and equipment (including software), together with the methods for bacterial maintenance and recovery, are given in Appendix A. All experiments described in this chapter employed *E. coli* R31, *E. coli* SB1, *E. coli* M23, *E. coli* MG1655, *L. monocytogenes* ATCC19115, *L. monocytogenes* ScottA and *L. monocytogenes* Fw03/0035.

2.3.2 General Methods

2.3.2.1 Preparation of Stationary Phase Populations of *E. coli*

Five colonies of each of the four *E. coli* strains grown on tryptone soya agar (TSA) were inoculated to separate 80ml tryptone soya broths (TSB) in a 250ml Erlenmeyer flasks and incubated statically at 37°C for 24(± 0.25) h, to achieve population densities of approximately 9.0 log colony-forming units (CFU) ml^{-1}.

2.3.2.2 Preparation of Stationary Phase Populations of *L. monocytogenes*

Five colonies of each of the three *L. monocytogenes* strains that had been grown on Brain Heart Infusion agar (BHA) were inoculated to separate 80ml Tryptone Soya Broths with 0.6% Yeast Extract (TSB-YE) in a 250ml Erlenmeyer flasks and incubated statically at 37°C for 24(± 0.25) h, to achieve stationary phase populations of approximately 9.0 log CFU ml^{-1}.

2.3.2.3 Preparation of Low a_w and Low pH Broth

To yield a final a_w of 0.900 (± 0.003) and pH of 3.50 (±0.05), TSB (for *E. coli*) or TSB-Ye (for *L. monocytogenes*) was prepared in less than 1000ml of distilled water in a volumetric flask, to which the appropriate amount of NaCl, determined by reference to Table 3 in Chirife and Resnik (1984), had been added. The medium was warmed to dissolve the salt and then sterile distilled water was added to make the final volume up to 1000ml. Broths were then autoclaved at 121°C, 106 kPa for 15 min. After cooling, the pH was aseptically adjusted to 3.50 (±0.05) using 10M HCl. Prior to each experiment, aliquots (50 ml) of the 1000ml broth stock were aseptically transferred into sterile 250 ml flasks. Flasks were incubated at the

appropriate temperature in a water bath for several hours prior to inoculation with test cultures to equilibrate to the required temperature.

2.3.2.4 Inoculation of Test Strains to Low a_w and Low pH Broth

Aliquots (5ml) of stationary phase populations of each bacterial strain were transferred to 15ml sterile tubes. Cells were pelleted by centrifugation at 1964 *g* for 15 minutes at room temperature (RT). The supernatant was removed by pipetting and the cell pellet was resuspended in 50ml of a_w (0.900) and pH (3.50) broth. Specifically, a 1ml aliquot of a_w (0.900) and pH (3.50) broth was removed from the flask and used to resuspend the cell pellet. This cell suspension was then returned to the flask, thus providing a 1/10 dilution of the original stationary phase population. Flasks were incubated at the required temperature (from 5°C to 45°C) in a water bath with shaking at 60 oscillations per minute. Immediately following inoculation, and at the end of the treatment, a 2 ml aliquot was withdrawn for a_w and pH measurement.

2.3.2.5 Enumeration of Viable Cells and Construction of Survival Curves of *E. coli* and *L. monocytogenes*

The viability of each population was estimated by culture-based enumeration immediately prior to the inimical treatment and either at regular intervals throughout or at specific time points as described. Specifically, 100µl aliquots were removed and serially diluted in diluent (0.1% of Bacteriological peptone and 0.85% NaCl). Samples (50 or 250µl volumes) were surface plated using a spiral plater onto brain heart infusion agar supplemented with 0.1% sodium pyruvate (BHA-P). Plates were incubated at 37°C for 14 (±0.5) h for *E. coli* and 24 (±0.5) h for *L. monocytogenes*, and CFU were quantified using an image scanner and CIA-BEN

software. Survival curves were constructed by plotting the logarithm (log) of the CFU.ml^{-1} against time. For convenience, when viable cells could not be detected in a 50µl volume of an undiluted sample, the number of viable cells was plotted as 1.3 log CFU. ml^{-1} (detection level). Where no colonies were detected, from a 250µl volume plated, the viable count was plotted as 0.6 log CFU. ml^{-1}.

2.3.2.6 Determination of Inactivation Rates and Evaluation of Differences between Species

Assuming log-linear inactivation kinetics, a set of 63 inactivation rate data was generated by the line of best fit to each curve for the range of conditions shown in Table 2.2. For each strain, temperature and inactivation rate data were transferred and were plotted as an Arrhenius plot [*ln* (inactivation rate) *vs.* 1/(absolute temperature)] to compare the effects of temperature on inactivation kinetics of the two species. Additionally, the mean inactivation rate and standard deviation for each organism was calculated for each temperature and differences between species were assessed by Student's t-test, using Microsoft® Excel. Differences were considered to be significant when $p>0.1$. The slopes, y-intercepts and R^2 of Arrhenius plots for each strain were determined by linear regression analysis using Microsoft® Excel.

2.4 RESULTS

2.4.1 *E. coli* and *L. monocytogenes* Inactivation Curves at Non-Permissive pH and a_w Level but at a range of Permissive Temperatures

The inactivation curves of the four strains of *E. coli* and three strains of *L. monocytogenes* at temperatures in the range 5°C to 45°C are shown in Figs B.1-9

(Appendix B). The inactivation responses for *E. coli* and *L. monocytogenes* were similar at temperatures less than 45°C (Fig B 1-8). These data suggest that there are no systematic differences in the inactivation rate of *E. coli* and *L. monocytogenes* at pH 3.5 and a_w 0.90 at temperatures in the range 5 to 40°C, despite that individual datasets show variability. At 45°C (Fig B 9), however, it is apparent that at pH 3.50 and a_w 0.900, the inactivation rates of *L. monocytogenes* are initially much faster, and more complex (apparently biphasic) than those of *E. coli* (Fig B9).

2.4.2 Arrhenius Plots

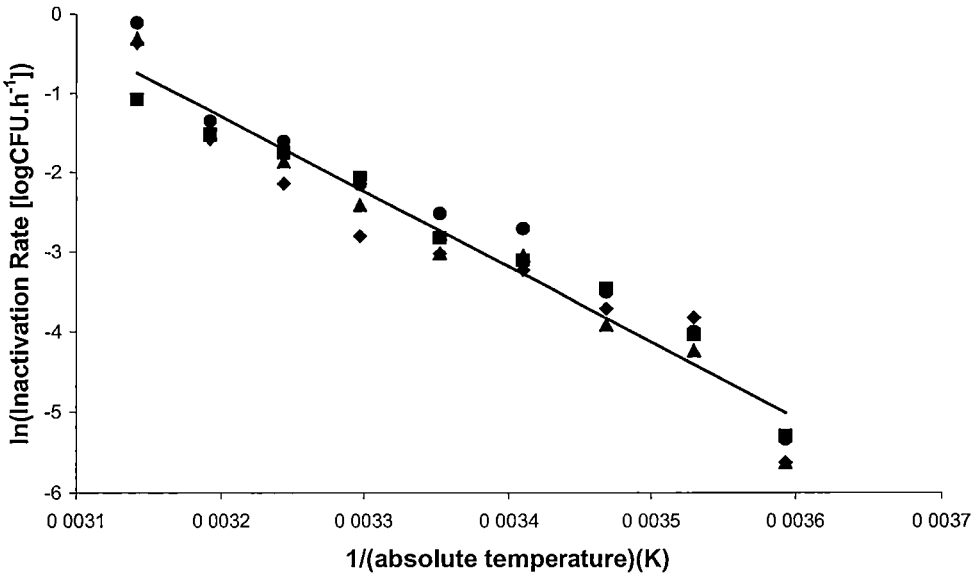
Given the focus of the thesis was to assess the degree of similarity responses of different vegetative bacteria to non-thermal, lethal, conditions, more complex curves that were observed in some cases, e,g,. *L. monocytogenes* ScottA at 45°C were initially assumed to represent uncontrolled experimental variation and were assumed to be essentially log-linear inactivation. Other studies (Brown, 2002; Shadbolt, 2004) suggest that this variation may be due to mixtures of exponential phase and stationary phase cells in the test population. Nonetheless, deviations from log linear kinetics are discussed subsequently. All the data were fitted to the Arrhenius equation by "brute force" as described in (Raisn, 1973; McMeekin et al., 1993). The inactivation rates calculated from each inactivation curve are given in Table 2.2 and are plotted on Arrhenius coordinates in Fig 2.2a for *E. coli* and Fig 2.2b for *L. monocytogenes*.

Table 2.2 Inactivation rates calculated from lines of best fit for all strains at all temperatures

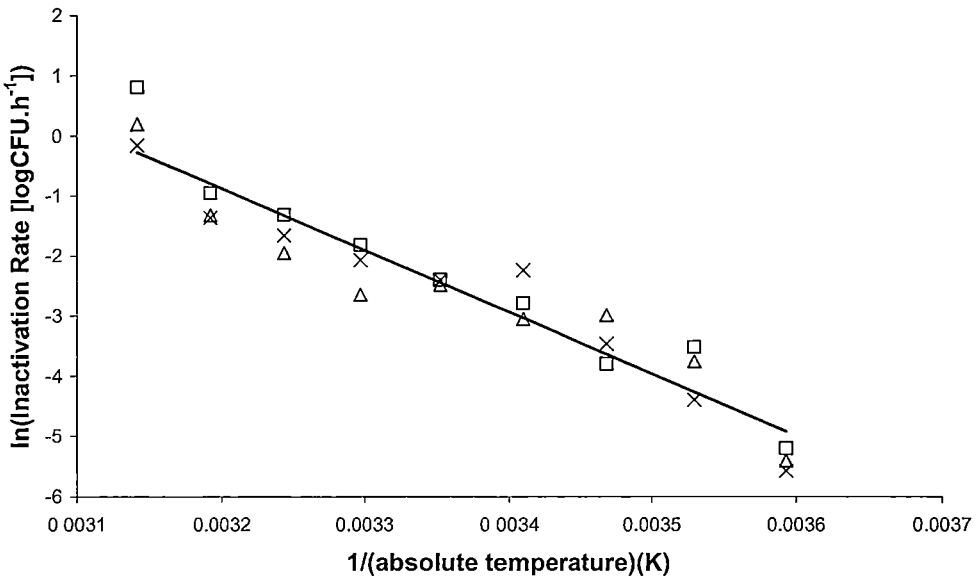
Temp (1/K)	Temp (°C)	*E. coli* strain				mean	S.D.(±)	*L. monocytogenes* strain			mean	S.D.(±)
		R31	SB1	M23	MG1655			ATCC19115	Scott A	Fw03/0035		
0.00359	5	-0.0050	-0 0036	-0.0036	-0.0048	-0.0043	0 0008	-0.0055	-0.0038	-0.0045	0.0046	0.0009
0.00353	10	-0.0176	-0.0145	-0.0218	-0.0183	-0.0181	0.0030	-0.0295	-0.0123	-0.0232	0.0217	0.0087
0.00347	15	-0.0314	-0.0199	-0.0244	-0.0300	-0 0264	0.0053	-0.0224	-0.0313	-0.0506	0.0348	0.0144
0.00341	20	-0.0448	-0.0473	-0.0396	-0.0667	-0.0496	0.0118	-0.0616	-0.1062	-0.0474	0 0717	0 0307
0.00335	25*	-0.0595	-0 0487	-0.0486	-0.0804	-0.0593	0 0150	-0 0910	-0.0901	-0.0837	0.0883	0.0040
0.00330	30	-0 1266	-0.0899	-0.0606	-0.1163	-0.0984	0.0295	-0.1617	-0.1257	-0.0708	0.1194	0.0458
0.00324	35	-0 1732	-0.1561	-0.1173	-0 2002	-0.1617	0.0347	-0.2676	-0.1897	-0.1419	0.1997	0 0634
0.00319	40	-0.2203	-0.2182	-0.2060	-0.2589	0 2259	0.0229	-0.3842	-0.2553	-0.2662	0.3019	0.0715
0.00314	45	-0.3400	-0.7348	-0.6947	-0.8939	0.6659	0.2336	-2.2465	-0.8531	-1.2157	1.4384	0.7229

S. D. Standard Deviation.

*n.s. denotes at the temperature are significantly different (p>0.1).



a

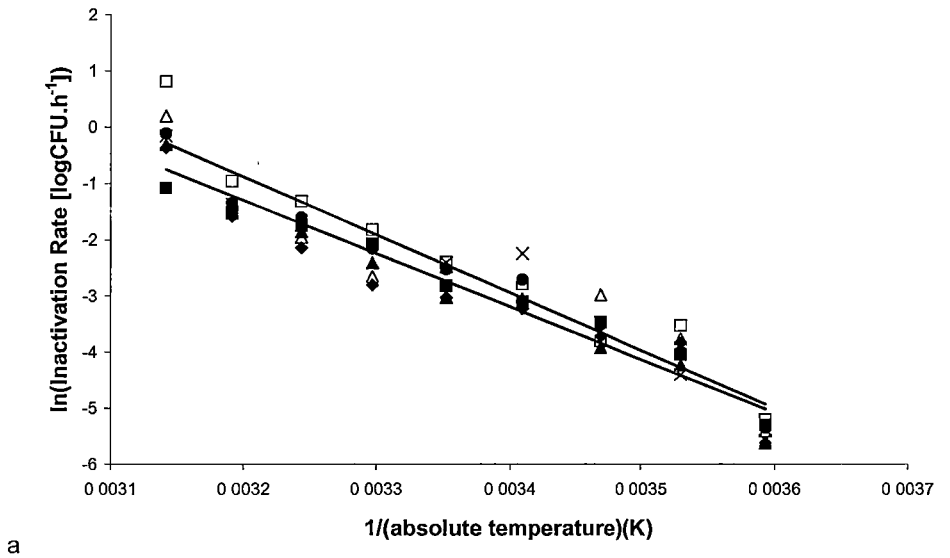


b

Fig 2.2 The effect of temperature on the rate of inactivation of a: *E. coli* R31 (■) *E coli* SB1 (▲) *E. coli* M23 (◆) and *E. coli* MG1655(●) [y=-9443.3x+28.919 R^2=0.9625] and b: *L. monocytogenes* ATCC19115 (□). *L. monocytogenes* ScottA (x) and *L. monocytogenes* Fw03/0035(Δ) [y=-10286x+32.037 R^2=0 9433] in a broth model at pH 3 50, a_w 0.900 and temperatures from 5°C to 45°C and 5°C interval. The data is shown as an Arrhenius plot [ln (inactivation rate) vs 1/(absolute temperature)].

It is clear that temperature has a strong effect on the rate of inactivation of *E. coli* and *L. monocytogenes* and the correlation coefficients (R^2) are 0.9625 and 0.9433,

respectively. The effect is very similar for both organisms under the inimical conditions employed, as shown in Fig 2.3a. When the data for *E. coli* (0-45°C) and *L. monocytogenes* (0-40°C) are compared separately, the lines of best fit for each organisms are parallel, as shown in Fig 2.3b.



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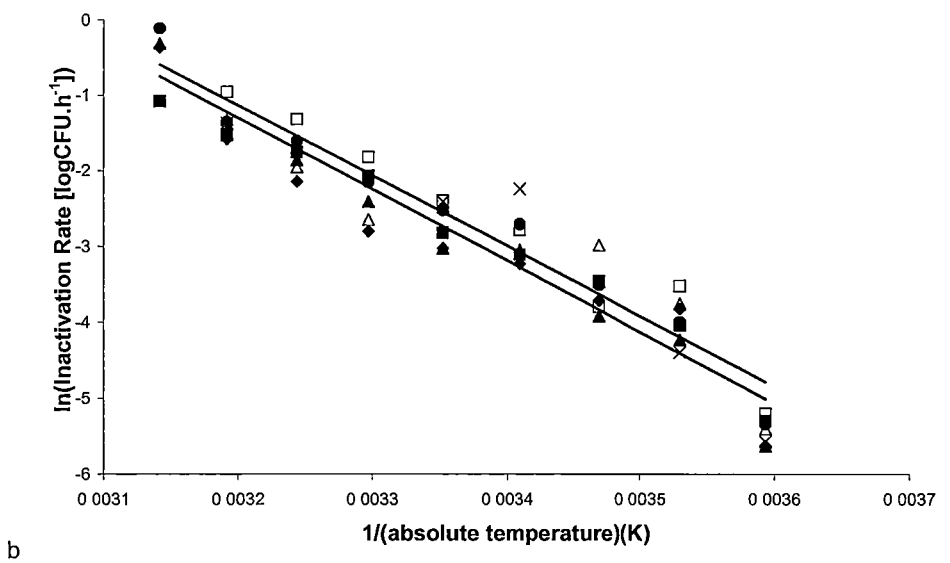


b

Fig 2.3 Comparison of the effect of temperature on the rate of inactivation of *E. coli* R31 (■), *E. coli* SB1 (▲), *E. coli* M23 (◆) and *E. coli* MG1655 (●) with *L. monocytogenes* ATCC19115 (□), *L. monocytogenes* ScottA (x) and *L. monocytogenes* Fw03/0035(Δ) at temperatures from a: 5°C to 45°C for both species and b: 5°C to 45°C for *E. coli* and 5°C to 40°C for *L. monocytogenes* [y=-9296.2x+28.616 R^2=0.9426] in a broth model at pH 3.50, a_w 0.900. The data is shown as an Arrhenius plot [ln (inactivation rate) vs 1/(absolute temperature)]. The lines are simple linear regressions fitted to the combined *E.coli* data and the combined *L.monocytogenes* data.

2.4.3 Similarities between Species

The slopes and intercepts of Arrhenius plots for individual strains are presented in Table 2.3. Differences in the mean inactivation rate for each species at a given temperature were tested for significance by Student's t-test, which indicated that there were no significant differences ($p<0.05$) between strains of both species at all temperatures tested except at 25°C (Table 2.2).

Table 2.3 Slope, *y*-intercept and correlation co-efficient of Arrhenius models for the rate of inactivation of *E. coli* and *L. monocytogenes* by pH 3.5 and water activity 0.90

Organism	Strain	Slope	y-intercept	R^2
E. coli	R31	-8588	26.1	0.97
	SB1	-10101	31.0	0.96
	M23	-9252	28 2	0.92
	MG1655	-9832	30.4	0.96
	Mean (±SD)	-9443 (±671)	28.9 (±2.4)	0.95 (±0.02)
L. monocytogenes	ATCC 19115	-11103	35.0	0.94
	Scott A	-10258	31.9	0.93
	Fw 03/0035	-9496	29.3	0.89
	Mean (±SD)	-10286(±804)	32.1 (±2.9)	0.92 (±0.03)
Mean (±SD) for both species		-9804 (±802)	30.3 (±2.8)	0.94 (±0 03)

The seven data sets are well described by the Arrhenius equation (average R^2 = 0.956). When the means of the slopes or intercepts of all *E. coli* strains were compared to that calculated for all *L. monocytogenes* strains there were no significant differences ($p<0.05$), indicating that the effect of temperature on the inactivation rate of both species is similar. The inactivation rate data for *L. monocytogenes* at 45°C was removed, due to the inconsistencies described above, and the line fitted to individual strains was forced to pass through the same intercept, calculated as the mean of all intercepts in the reduced data set (i.e. *ln* inactivation

rate = 30.3). The resultant mean slopes for the four *E. coli* strains and the three *L. monocytogenes* strains were -9443, -10286 (5-45°C) and -9296 (5-40°C), respectively, and are not significantly different ($p<0.05$). When linear regression analysis was applied to the four *E. coli* strains and the three *L. monocytogenes* strains, the effect of temperature was very similar for both organisms under the inimical conditions employed, especially when the data for *L. monocytogenes* inactivation at 45°C were omitted, as shown by the slope and *y*-intercept values given in Table 2.4.

Table 2.4 Comparison of the slope, *y*-intercept and correlation co-efficient of Arrhenius models for the rate of inactivation of four strains of *E. coli* and three strains of *L. monocytogenes* by pH 3.5 and water activity 0.90.

Organism	Temp. range (°C)	Slope	y-intercept	R^2
E. coli	0-45	-9443	28.9	0.95
L. monocytogenes	0-45	-10286	32.1	0.92
L. monocytogenes	0-40	-9296	28.6	0.91

2.5 DISCUSSION

Previous investigations (McQuestin, 2006; Ross et al., 2004; Ross and Shadbolt, 2001) indicated that temperature is the main factor governing the inactivation rate of *E. coli* in fermented meat products or other growth preventing environments. Fermented meats are characterised by water activity and pH values that, during fermentation and maturation, become increasingly inimical to *E. coli* growth, eventually preventing it. Whereas the temperatures during manufacture and storage are non-lethal, they appear to strongly influence the amount of *E. coli* inactivation that occurs during these manufacturing processes. These experiments were initiated

to test the generality of this apparently dominant effect of non-lethal temperature by determining if the same phenomenon occurs in the Gram-positive bacterium *L. monocytogenes*. The results presented in this chapter reveal a striking similarity in the inactivation response to non-lethal temperature of these two species when other hurdles preclude growth.

2.5.1 Comparison of *E. coli* and *L. monocytogenes* Inactivation Kinetics

The results show that at temperatures from 5 to 40°C, the kinetics of inactivation of four strains of *E. coli* and three strains of *L. monocytogenes* in response to a_w 0.90 and pH 3.5 were very similar at each temperature (Fig B1-8). However, at 45°C (Fig B9), the rates of inactivation of the *L. monocytogenes* strains were initially faster than that for the *E. coli* strains. *L. monocytogenes* has an upper growth limit of 45°C (Junttila et al., 1988b; Ross et al., 2000, see Table 2.1). Therefore, the temperature of incubation in these experiments may have been lethal, in itself, to *L. monocytogenes* and the greater rate of decline in the population (Fig B 9) may have been due to the combined effect of temperature above the maximum that allows growth, pH and a_w. The comparison of the two lines fitted to the *E. coli* and *L. monocytogenes* inactivation rate data with and without 45°C data for *L. monocytogenes* included (Fig 2.2), gives a direct view of the influence of the three inactivation rate data of *L. monocytogenes* at 45°C. It is apparent that the two lines are virtually parallel after omitting those three data points that potentially represent thermal inactivation of *L. monocytogenes*. Furthermore, in Table 2.4, the Arrhenius equation fitted to the *E. coli* data set was compared to that using the full *L. monocytogenes* data set (i.e. for 5-45°C) and, a reduced data set with the 45°C data

omitted. With the removal of the 45°C data for *L. monocytogenes*, the effect of temperature explained 95% and 91% of the observed *ln* (inactivation rate) data for *E. coli* and *L. monocytogenes*, respectively. The slopes of the Arrhenius plots fitted to the *E. coli* (0-45°C) and *L. monocytogenes* (0-40°C) data are almost identical and the *y*-intercepts of these models are very similar. This effect is an example of hurdle technology (Leistner, 2000b, Leistner, 1994), where several sub-lethal stresses combined can cause cell inactivation.

As noted, Table 2.2 shows that, at temperatures in the range 5 to 45°C, the rates of inactivation of four strains of *E. coli* and three strains of *L. monocytogenes* in response to pH 3.5 and a_w 0.90 are not significantly different at any temperature tested, with the exception of 25°C. Examination of inactivation kinetics generated at 25°C (Fig B 5), however, suggests that this statistical difference may be due to unusually consistent inactivation rates of the three *L. monocytogenes* strains at this temperature, for which the standard deviation is approximately an order of magnitude less than at other temperatures (Table 2.2). Indeed, the rate of inactivation of *E. coli* MG1655 at 25°C is more comparable to the inactivation rates for the three *L. monocytogenes* strains than it is for the other *E. coli* strains at that temperature. Therefore, there appear to be no systematic differences in the inactivation rates of *E. coli* and *L. monocytogenes* at 5 to 45°C by pH 3.5 and a_w 0.90 despite that inactivation rates at specific temperatures show variability.

2.5.2 Assessment of the Hypothesis

The hypothesis to be tested was that the effect of temperature on non-thermal inactivation of vegetative bacteria is similar irrespective of species. Figs B1-8 show highly similar inactivation kinetics between *E. coli* and *L. monocytogenes*.

Inactivation rates, except at 25°C as discussed above, shows no significant differences ($p<0.05$) between strains of either species (Table 2.2). Fig 2.3b shows that inactivation rates of either species are very similar. Furthermore, there were no significant differences ($p<0.05$) between the means of the slopes or intercepts of *E. coli* and *L. monocytogenes* (Table 2.3). At any temperature tested Table 2.4 further accentuates the similarities in inactivation kinetics of the two species. Overall, these results indicate that the inactivation of *E. coli* and *L. monocytogenes* by pH 3.5 and a_w 0.90 is strongly influenced by non-lethal temperature and that the quantitative and qualitative effect of temperature in each case is highly consistent, despite that growth responses of *E. coli* and *L. monocytogenes* to water activity and pH are different (Table 2.1).

2.5.3 The Influence of Media

Fig 2.4 compares the Arrhenius models of McQuestin (2006), based on *E. coli* inactivation, with the models developed in this study using *E. coli* and *L. monocytogenes* in nutrient broths that prevent growth. A simple linear regression is fitted to the combined data for both species apart from 45°C data of *L. monocytogenes*. Combining the data for all seven strains gave an R^2 for the fitted equation of 0.9625, indicating that the effect of temperature explains 96% (similar to the R^2 of the four strains of *E. coli* on an Arrhenius plot alone, as Fig 2.2a shows) of the variance in the observed *ln* (inactivation rate) data. The other black line is fitted to the data of McQuestin (2006) for inactivation of *E. coli* M23 at a larger range and variety of environmental factors in broth designed to imitate conditions in salami. These two lines appear to be parallel. The relative effect of temperature, i.e. the slope of the Arrhenius plot, is highly consistent between the model derived in

this study for *E. coli* and *L. monocytogenes* and the other model for *E. coli*, while differing significantly in absolute rate.

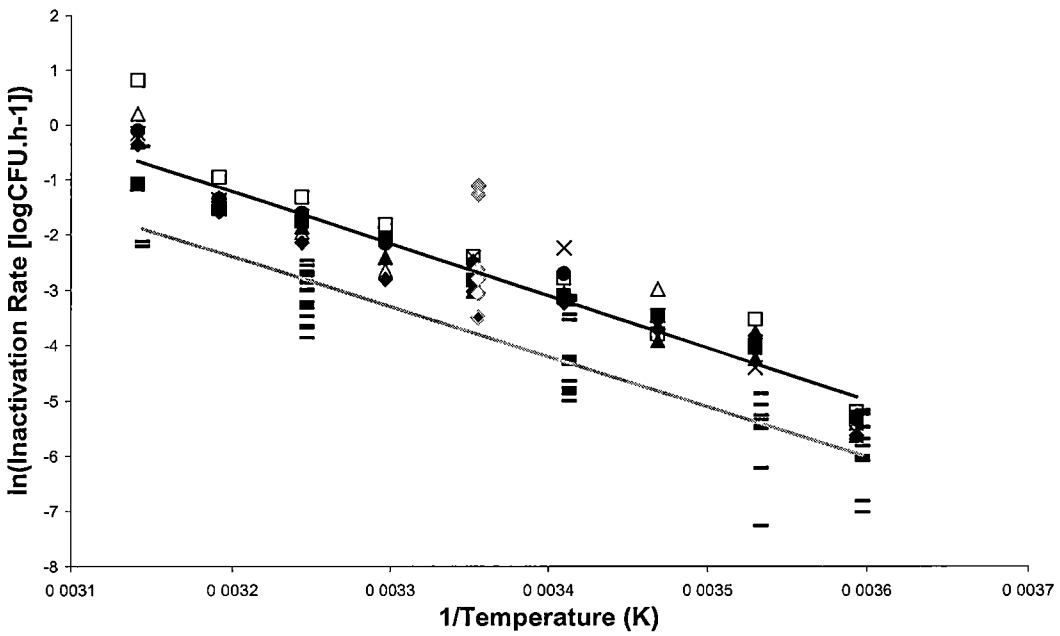


Fig 2.4 Comparison of the effect of temperature on the rate of inactivation of *E. coli* (5°C to 45°C) and *L. monocytogenes* (5°C to 40°C) in a broth model at pH 3 50, a_w 0.900 (this study [black line· y = -9440.4x + 28.997 R^2 = 0.9625]) with *E. coli* M23 (−) ın salami [grey line: y = -9052.1x + 26.575 R^2 = 0.8104] and *E. coli* M23 (◇) in the normal broth model at various combinations of a_w, pH, lactic acid concentration and oxygen availability (McQuestin, 2006). The data is shown as an Arrhenius plot [ln (ınactivation rate) vs 1/(absolute temperature)].

Ross et al. (2004) reported that the slopes of the lines of best fit on Arrhenius plots for a data set of *E. coli* inactivation in fermented meat and another in a broth-based system designed to mimic the physico-chemical conditions in fermented meats overlapped. In contrast, in this study, the lines of best fit of two independently derived data sets appear parallel but with a "gap", as shown in Fig 2.4. Especially, *E. coli* inactivation appears slower in fermented meats than in analogous broth. To explain, in Fig 2.4, the grey diamond points, which are well described from the

model of this study, are based on McQuestin's (2006) data, derived from studies in a broth medium. Thus, there appears to be a systematic difference in rate of inactivation observed in studies using fermented meats and those derived from broth. The rates of inactivation are apparently slower in fermented meats than in broths.

A potential explanation may be due to the complexity of the environment to which the vegetative bacteria were exposed. The significance difference in inactivation rates (Fig 2.4) in broths and fermented meats might be a result of the difference in environments. McQuestin et al. (2006) found that differences in inactivation rate resulted from use of different suspending media including inimical media and a range of complex nutrient-rich media. Other data collected by Ross et al. (2004) in either a fermented meat or a broth-based system designed to mimic the physico-chemical conditions of fermented meats, also revealed systematic differences in inactivation rates, with inactivation being consistently faster in inimical broths than in analogues fermented meat environments , as were used in this study.

2.5.4 The Lack of Influence of Other Factors

Table 2.5 compares the results of the current study to those previously reported. Although the media, species strains and conditions are different, R^2 was greater than or equal to 0.66 in all cases, which indicates that temperature alone explains over 66% of the variance in the observed *ln* inactivation rate data. While temperature is an important factor governing the inactivation of vegetative bacteria, other factors clearly influence the rate of inactivation.

Table 2.5 Comparison of published data with data from this study

Author and Year	Medium	Bacteria species	R^2	Equation
Ross and Shadbolt (2001)	UCFM	*E.coli*	66%	Y=-11255x+33.378
McQuestin (2006)	UCFM	*E.coli*	81.04%	Y=-9052.1x+26.575
Current study	Broth	*E.coli & L.mono*	96.25%	Y =-9440.4x+28.997

Ross and Shadbolt (2001) attempted, as far as possible from the existing literature, to discern and quantify the patterns of *E. coli* inactivation in environments relevant to UCFM products and processes. The data from a variety of published and unpublished sources were collated and inactivation rates calculated. In many cases the inactivation data was not ideal, e.g. few points, multiphasic inactivation rates, different strains, different methods, few points from which to estimate inactivation rates, etc. Data were drawn from experiments both in fermented meat products and in various types of broth systems, and the change in numbers of surviving *E. coli* with time used to estimate inactivation rates (Ross et al., 2004). As a result, the influence of temperature accounts for 66% of the variability in that dataset.

McQuestin (2006) developed a broth model to imitate the environment of UCFM, and studied *E. coli* M23 inactivation rates at various combinations of temperature, water activity, pH, lactic acid concentration and oxygen availability. The simplicity of the media, the rigorous collection of the data and consistent estimation of the inactivation rates, resulted in r^2 values equal to 0.8104, indicating that the effect of temperature explains 81% of the variance in the observed *ln* (inactivation rate) data in that experimental system.

In the current study, using a well-controlled experimental system, the independent variables, i.e. species and strain of bacterium, were found to contribute little to the variability in response since the effect of temperature explained over 90% of variance in the *ln* inactivation rate data for both *E. coli* and *L. monocytogenes* when exposed to pH 3.5 and a_w 0.90. This study indicates that bacterial strain and species are not particularly important determinants of non-thermal inactivation rates. In other words, the factors that might weaken the temperature influence appear to be experimental techniques or the extent of the complexity of the other environmental factors rather than the microorganism species or strains. Therefore, it is suggested that the influence of non-lethal temperature on the rate of inactivation of vegetative bacteria in inimical environments is not species-dependent.

2.6 CONCLUSION

The main aim of this study was to determine whether temperature is not only the greatest influence on inactivation of *E. coli*, a Gram-negative organism in inimical environments, but whether that observation is also true of other vegetative bacteria. Thus, *L. monocytogenes*, as a Gram-positive organism, was selected because its physiology differs from that of *E. coli*. The rates of inactivation of four strains of *E. coli* and three strains of *L. monocytogenes* in brain heart infusion broth at pH 3.5, water activity 0.90 and at the temperature range 5-45°C (at 5°C intervals) were determined. For temperatures below the maximum for growth, i. e. 5°C to 40°C, there were no systematic differences in the inactivation rates of *E. coli* and *L. monocytogenes*, despite that individual data sets showed variability. These results support the hypothesis that temperature is the prime factor governing the

inactivation of vegetative bacteria when they are prevented from growth by other, non-thermal, environmental factors. To extend the generality of the observation and further test the hypothesis, investigation is needed on other non-thermal stresses, bacterial species and applied on other food products such as cheese. This will be very useful for understanding safety, processing and preservation of non-thermal processed foods, such as salami.

CHAPTER 3

TESTING INTRACELULLAR ATP LEVEL AS A RAPID METHOD FOR ASSESSING MICROBIAL INACTIVATION

3.1 ABSTRACT

Plate count based enumeration methods for detecting microbial inactivation are labour and space intensive and slow. To investigate more sensitive and rapid methods for assessing inactivation of microbial populations in broth culture, measurement of intracellular ATP (adenosine 5'-triphosphate) using luminometry was assessed. *L. monocytogenes* ScottA and Fw 03/0035 were inactivated by exposure to inimical conditions of pH 3.50 and a_w 0.90 at temperature 25°C, 35°C and 45°C, respectively. Samples were periodically withdrawn for enumeration by parallel methods, i.e. viable count and cellular ATP content. The results showed that the ATP method is not comparable to the viable count method and the inactivation rates and kinetics are totally different, especially at 25 and 35°C. To further investigate the utility of the ATP method, a growth study was also undertaken. Cells were grown from 10^4 CFU ml^{-1} to stationary phase in TSB-Ye broth at 25°C. The results demonstrate a good correlation for growth rate assessed periodically by viable count and ATP methods. From this study it is concluded that ATP measurement is not sensitive enough to enumerate microbial inactivation, particularly when cells are inactivated in non-thermal lethal conditions; however, it is well correlated with microbial growth.

3.2 INTRODUCTION

Rapid methods in microbiology are dynamic fields of study that address the utilisation of microbiological, chemical, biochemical, biophysical, immunological, and serological methods for the study of improving isolation, early detection, characterisation, and enumeration of microorganisms and their products in clinical, food, industrial, and environmental samples (Fung, 2000).

Many rapid methods, taking around an hour or less, have been developed in recent years to estimate the total number of microorganisms by parameters other than the viable colony count (Stanley, 1989), and one such rapid technique involves measuring cellular ATP utilising firefly luciferase induced bioluminescence. The amount of luminescence produced can be directly related to microbial numbers and is measured with a luminometer within minutes.

All cells utilise ATP. Within a microbial cell, ATP concentration plays a special role, not only as an energy source but also as a regulator of the activity of many enzymes, such as an intermediate carrier of chemical energy linking catabolism and biosynthesis (Kennedy and Oblinger, 1985). The production of ATP, however, should be viewed not as a mechanism for the storage of chemical potential energy, but rather as a system for rapid and specific mobilization of cellular energy (Hobson and Summers, 1972). Careful studies have shown that the cellular level of ATP does change to some extent. Thus there have been reports that certain factors change the ATP level to diverse extents (Stanley, 1986) including: cell division, growth cycle, pH, temperature, nutrients (including O_2), inhibitors, antibiotics and biocides. However, for a given set of intra- and extra-cellular conditions ATP

remains relatively constant. Thus, the average cellular ATP level in a population of non-synchronous microbial cells is a good index for estimating and enumerating those cells (Baker *et al.*, 1992).

For a new method to be considered acceptable for assessing microbial inactivation, it must fulfil certain criteria to be a useful and appropriate estimate of living biomass, which means it should have some direct correlation with the total viable cell count. For example, the measured quantity should be proportional to some cellular entity, and there should also be a sensitive and accurate analytical procedure available to measure the parameter. The ATP method fulfils these criteria and the mechanism of the bioluminescence assay, mediated by the luciferin/luciferase reaction for ATP, has been well documented (Champiat et al., 1994; Silley, 1994; Stewart and Williams, 1992). In the presence of a firefly enzyme system (luciferase and luciferin system), oxygen and magnesium ions, ATP from living cells will facilitate the reaction to generate light. The small amount of light signal produced is proportional to the amount of ATP present in the sample and thus microbial number (Stannard and Wood, 1983). The light emitted by this process can be monitored by a variety of luminometers.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial Strains, Media, Reagents, Solutions and Equipment

Details of bacterial strains, bacteriological media, chemical reagents, solutions and equipment (including software), together with the methods for bacterial maintenance and recovery, are given in Appendix A. All experiments described in this chapter employed *L. monocytogenes* ScottA, a type strain extensively used in

experimental studies, and *L. monocytogenes* Fw03/0035, a high salt resistance strain.

3.3.2 "ATP"-Based Rapid Enumeration Method

3.3.2.1 Extraction of ATP (5% or 10% TCA Extraction Comparison)

For the extraction of adenylate from each sample, a 1ml aliquot of a population of *L. monocytogenes* (see Section 2.3.2.2) was added to 1ml of ice-cold TCA/EDTA solution (either 5% or 10%/4mM) with 0.0005% to 0.002% of xylenol blue dye for 10 min. In this state the mixture was stable and could be stored in the refrigerator for a short time or in the freezer for a long period of time until all of the samples were ready for analysis. Acid precipitable material was then removed by centrifugation at room temperature (RT) and 3824 *g* for 10 min. The supernatant (containing extracted adenine nucleotides) was adjusted with 2M KOH to pH: 7.75 (the optimum pH for the reaction).

3.3.2.2 Construction of ATP standard curve

100µl aliquots of ATP standard (10^{-7}M) from ENLITEN® ATP Assay Kit was serially diluted to 10^{-12}M and made up to 1ml by ATP Assay Buffer (20mM Tris/2mM EDTA, pH7.75) in a disposable cuvette. ATP was then measured by automatic injection of 10µl luciferin-luciferase mixture, following light emission to the peak value, using a Berthold luminometer (see Section 3.3.2.4). A new ATP Standard curve was prepared each day that assays were performed, or whenever a new aliquot of the rL/L Reagent (ENLITEN® ATP Assay Kit) was used.

3.3.2.3 Preparation of samples

Serial dilution of stationary phase population

A 1ml aliquot of culture (stationary phase populations of *L. monocytogenes*, Section 2.3.2.2) was serially diluted in ATP Assay buffer, centrifuged at RT and 3824 *g* for 10 min. The supernatant was removed by pipette and ATP was extracted from the pellets with 10% TCA (Section 3.3.2.1).

Inactivation in Different Conditions (25, 35, 45°C with pH 3.5 and a_w0.9)

Immediately prior to the inimical treatment and either at regular intervals throughout or at a specific time point as described, 1ml aliquots of samples were removed and centrifuged at RT and 3824 *g* for 10 min. The supernatant was removed using a pipette and the cells were resuspended by transferring to a 1ml aliquot of TSB-YE. The resuspended aliquots were centrifuged at RT and 3824 *g* for 10 min. The supernatant was removed by pipette and ATP extracted from pellets with 10% TCA extraction (see Section 3.3.2.1).

3.3.2.4 Performing the ATP Assay

Adenine nucleotides were assayed with rL/L reagent (ENLITEN® ATP Assay Kit) using an Autolumat Berthold Luminometer. 100µl aliquots of extract of the supernatant contains extracted nucleotides were made up to 1ml using ATP Assay buffer (20mM Tris/2mM EDTA, pH7.75) in a disposable cuvette. ATP was then assayed by automatic injection of 10µl luciferin-luciferase mixture and following the light emission to the peak value. Relative Light Units (RLU) were quantified by

the Berthold Luminometer and the results printed out. Survival curves were constructed by plotting the logarithm$_{10}$ (log) of the RLU.ml^{-1} against time.

3.3.3 Growth Investigation

3.3.3.1 Preparation of Exponential Phase Populations of *L. monocytogenes*

Exponential phase populations of *L. monocytogenes* were prepared by transferring five colonies from BHA-P to 80ml TSB-YE in a 250ml Erlenmeyer flask and incubating statically at 37°C for 24 h, to a population density of approximately 9.0 log CFU ml^{-1}. The populations were diluted to ~10^2 CFU ml^{-1} in 80ml TSB-YE in a 250ml Erlenmeyer flask and incubated at 25°C until just visibly turbid (typically 30 h), which correlated to a population density of approximately 7.0 log CFU ml^{-1}. At this time, populations were diluted to ~10^4 log CFU ml^{-1} in 80ml TSB-Ye and incubated at 25°C until just visibly turbid (typically 15 h) providing a population density of approximately 7.0 log CFU ml^{-1}.

3.3.3.2 Enumeration of Viable Cells and Construction of Growth Curves of *L. monocytogenes* by Plate Count

100µl aliquots were serially diluted (0.1% of Bacteriological peptone and 0.85% NaCl) and 50 µl volumes were surface plated using a spiral plater onto BHA-P. Plates were incubated at 37°C for 24 (±0.5) h, and CFU were quantified by using an image scanner and CIA-BEN software. Survival or growth curves were constructed by plotting log CFU.ml^{-1} against time.

3.3.3.3 Optical Density based Enumeration Method

1ml aliquots of log phase populations of *L. monocytogenes* were transferred to 50ml of TSB-YE broth in 250ml sidearm flasks, and were incubated at the required temperature (25°C) in a water bath with shaking at 60 oscillations min^{-1}. Immediately after inoculation and either at regular intervals throughout or at a specific time point as described, the sidearm flasks which contained the culture were removed from the water bath and Optical Density (OD) at 600nm of the culture determined by a spectrophotometer. Growth curves were constructed by plotting $\log_{10}$ (OD) against time.

3.3.3.4 Determination of Growth Rates

Assuming log-linear growth kinetics, the growth rates of *L. monocytogenes* ScottA and Fw 03/0035 and the y-intercept were calculated for each growth curve by linear regression analysis using Microsoft® Excel. A set of 6 growth rate data were generated by the selected exponential phase time points, and were determined by the line of best fit to each curve for the three different methods: viable count, ATP method and Optical Density method.

3.3.4 Comparison of intracellular ATP levels as a rapid enumeration method with plate count and optical density

The viability of bacterial populations, either growing or being inactivated, were estimated by three methods in parallel. Immediately after inoculation and either at regular intervals throughout or at specific times as described, two 1ml aliquots were removed. From one aliquot, 100µl was removed for the traditional culture based

enumeration method (Section 2.3.2.5), and the other 1ml aliquot was used immediately for the ATP based rapid enumeration method (Section 3.3.2). At the same time, OD was measured using a spectrophotometer (Section 3.3.3.3).

3.3.4.1 Boiling test

For the last two samples taken from inactivation trials and one sample from a growth investigation, at the same time points, additional 1ml aliquots of the culture were removed from the flasks. The aliquots were immediately heat treated in a 100°C water bath for 5 min, and then centrifuged at RT and 3824 *g* for 10 min. The supernatant was removed using a pipette and the cells were resuspended by transferring to a 1ml aliquot of TSB-Ye. The resuspended aliquots were centrifuged at RT and 3824 *g* for 10 min. The supernatant was removed by pipette and the pellets were subjected to ATP extraction (Section 3.3.2.1) with 10% TCA.

3.4 RESULTS

3.4.1 ATP Standard Curve

As a new ATP standard curve must be freshly prepared daily, an example of standard curve generated with the Autolumat Berthold Luminometer is shown in Fig 3.1. ATP standard (10^{-7} mol l^{-1}) from ENLITEN® ATP Assay Kit was serially diluted to 10^{-12} mol l^{-1} and then measured by light emission following the protocol given in Section 3.3.2.4. Log RLU was plotted against log (moles of ATP). Theoretically, ATP standard should be diluted from 10^{-7} through 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11} to 10^{-12} mol l^{-1}, potentially, introducing error during serial dilution. However, in Fig 3.1, the relationship between log RLU and log moles of ATP was linear and

the R^2 of simple linear regression is 99.85%, and demonstrates the accuracy and sensitivity of the bioluminescent method over a very wide range of concentrations.

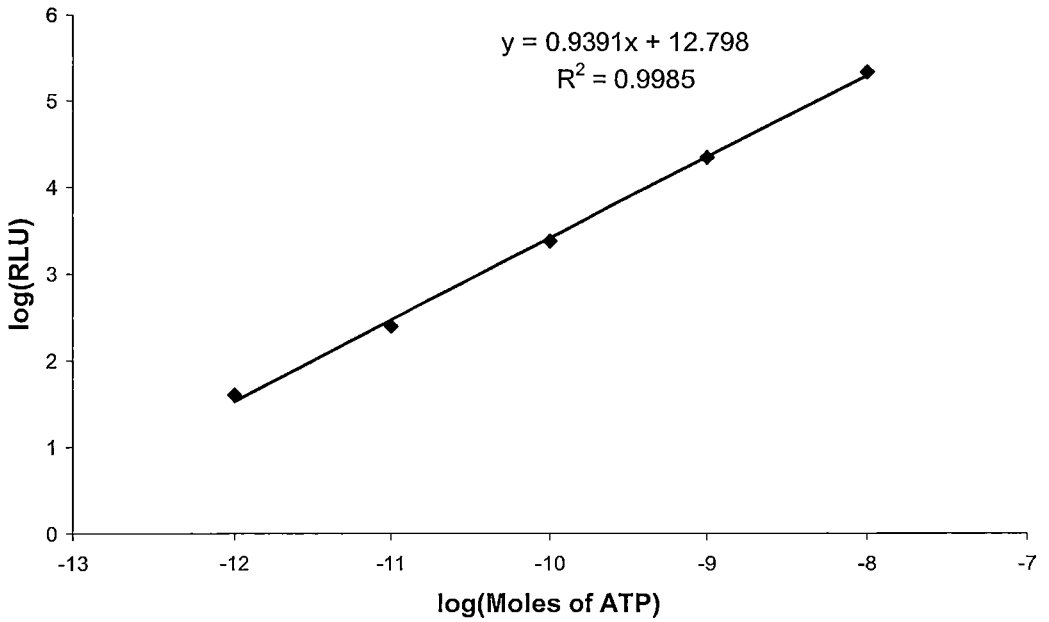


Fig 3.1 ATP standard (10^{-7}M) from ENLITEN® ATP Assay Kit was serial diluted 10^{-12}M in assay buffer (20mM Tris/2mM EDTA, pH7.75). ATP was then measured by light emission to the peak value by luminometry. A simple linear regression was fitted to the data. The correlation coefficient (R^2) is 99.85%.

3.4.2 ATP Extraction Selection (5 or 10% TCA)

The efficacy of either 5 or 10% TCA extraction of ATP was assessed using serially diluted stationary phase populations of *L. monocytogenes* ScottA (Fig 3.2). Assuming that 0 dilution represents 10^8 CFU. ml^{-1}, then, theoretically, five ten fold dilutions results in a suspension of 10^3 CFU. ml^{-1}, which is the reported sensitivity limit of the ATP bioluminescence method. The relationship between RLU emitted and *L. monocytogenes* numbers was linear (R^2=0.9983) for cells extracted using 10% TCA. In contrast, for extractions with 5% TCA, RLU was poorly correlated at

high population densities with relatively low emission (i.e.10-fold through 1000-fold dilutions). For 100-fold dilutions, the log RLU estimate obtained using 5% TCA extraction is ten fold lower compared with 10% TCA, and for the ten fold dilutions, underestimates cell density 100-fold compared to 10% extraction method. Thus, a 10% concentration of TCA was selected for the subsequent standard extraction method.

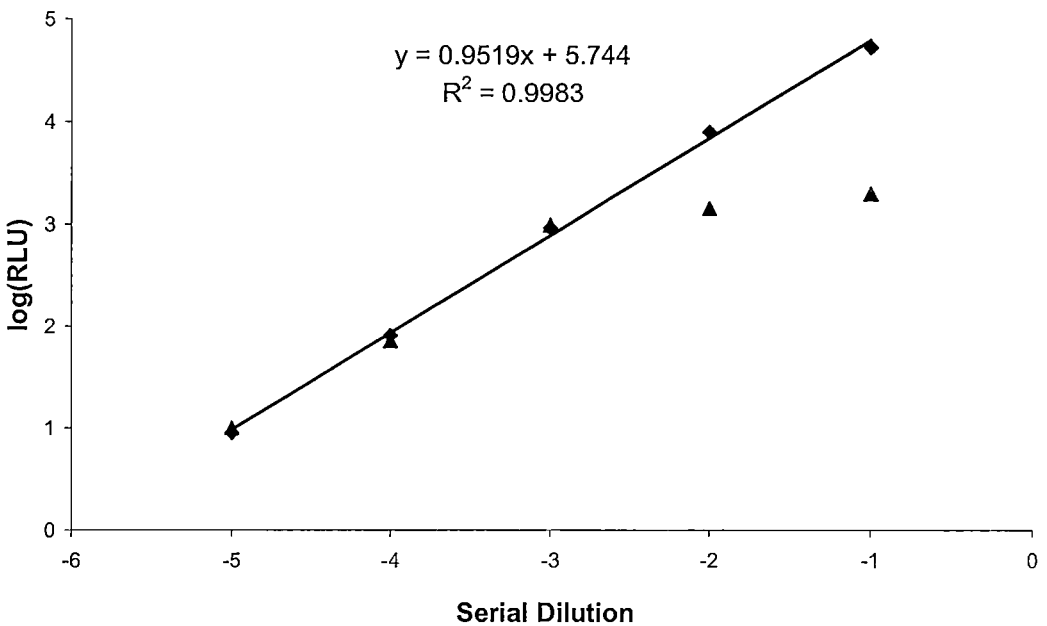


Fig 3.2 Stationary phase populations of *L. monocytogenes* ScottA were serially diluted. Each dilution was treated to extract adenine nucleotides using both 5% TCA (▲) and 10% TCA (◆) and ATP measured by bioluminescence For the 10% TCA extraction a linear regression to the data provides an excellent description (R^2 = 99.83%).

3.4.3 Parallel Study of *L. monocytogenes* Inactivation

The data for inactivation of exponential phase populations of *L. monocytogenes* ScottA and Fw 03/0035 are presented in Fig 3.3. Cells were inactivated by the inimical conditions of pH3.50 and a_w 0.90 at three different temperatures: 25°C,

35°C and 45°C, with samples withdrawn for parallel study of viable plate count and ATP method. From Fig 3.3 we can see that the viable count and ATP method are not correlated. The viable count data show that log linear inactivation commenced immediately upon exposure to the stress at 10^8 CFU.ml^{-1} and cells were no longer detectable after 215.5 h incubation. In contrast, the ATP level of both strains declined around 1 log in 25 h and thereafter declined gradually by another log for the remainder of the experiment. Even when there were no colonies growing on the viable count plates there was still light emission at the last three time points: 215.5, 240 and 264 h, suggesting that some cells may remain metabolically active but are not be able to form colonies on plates.

When cells were exposed to lethal conditions at 35°C (Fig 3.3b), they lost their ability to produce colonies on plates 10 and 16 h after the stress was imposed, for *L. monocytogenes* ScottA and Fw 03/0035, respectively. Conversely, the ATP level of both cultures decreased 1.5 log in 6 h and remained stable thereafter. Thus, a similar phenomenon happened as observed at 25°C, when cells still retained a certain level of ATP production, despite being unable to produce colonies on BHA-P agar, at the time points: 16, 23, 27 and 31 h.

Fig 3.3c shows the inactivation of cells under inimical conditions of 45°C, a temperature which is, of itself, lethal for *L. monocytogenes*. Viable cells numbers decreased from 10^8 to less than detection level within 1 hr of the treatment, while the intracellular ATP level declined by 2 log in 1 hr and then remained constant for a further four hours. The remaining level of luminescence is, however, nearly the same as the blank for the 35 and 45°C treatments, showing 0.5 and 1.5 log

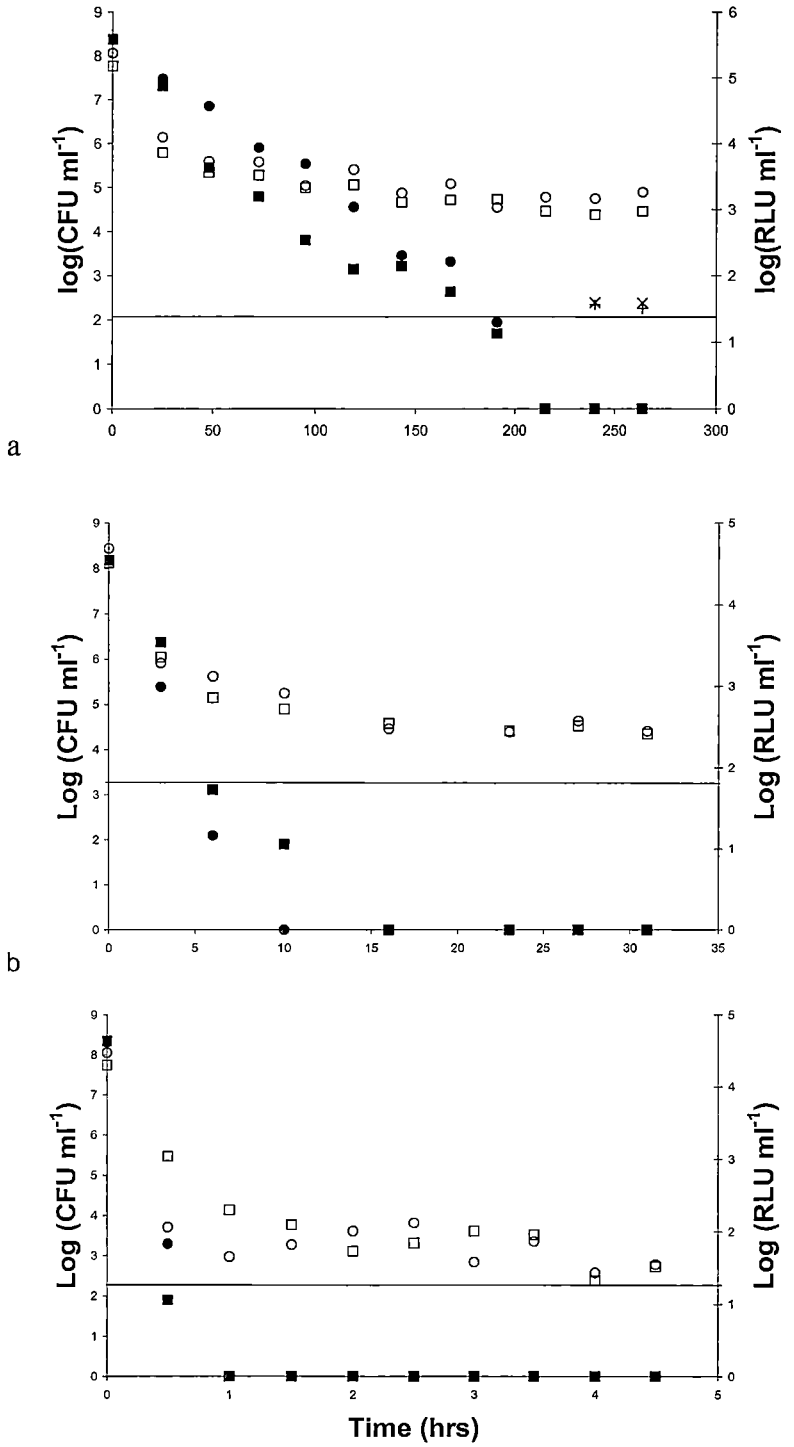


Fig 3.3 Exponential phase populations of *L. monocytogenes* ScottA and Fw 03/0035 were held in TSB-Ye broth under the conditions pH3.50, a_w 0.90, and at **a**: 25°C, **b**: 35°C, **c**: 45°C. Samples were periodically withdrawn for parallel assessment of viable count [*L. monocytogenes* ScottA (•) and Fw 03/0035(■)] and ATP content [*L. monocytogenes* ScottA (o) and Fw 03/0035(□)]. For the very last two samples at 25 °C, cells were boiled at 100°C for 5 min and followed by ATP method [*L. monocytogenes* ScottA (+) and Fw 03/0035(×)] The grey line is blank control.

differences, respectively.

Thus, a further experiment was undertaken to investigate this phenomenon. The last two samples of both strains at 25°C were boiled. While they could not grow on BHA-P plates, they still produced a luminescence signal. The results show that the intracellular ATP level of the boiled cells is about the same as the reagent blank but is lower than inactivation samples by over 1.5 log.

3.4.4 Parallel Study of *L. monocytogenes* Growth

A parallel study with growth, rather than inactivation, was undertaken for *L. monocytogenes* ScottA and Fw 03/0035 in TSB-YE broth at 25°C, as shown in Fig 3.4. Growth of both strains was determined by viable count, ATP method and optical density method.

As can be seen from Fig 3.4a, cells inoculated at 10^4 CFU.ml^{-1}, stayed in lag phase for 2 h before commencing exponential growth, and at time point 16 h, they began to enter stationary phase (10^8 CFU.ml^{-1}), and thereafter remained stable in number for the rest of the experiment. The intracellular ATP level of the cell population underwent a nearly parallel increase. The ATP level of both strains started at 10^2 RLU ml^{-1}, followed by lag phase for 2 h until exponential phase commenced, and 16 h later achieved stationary phase with a peak of 10^5 RLU. ml^{-1}. The RLU dropped dramatically, however when growth ceased: RLU for *L. monocytogenes* Fw 03/0035 decreased by 1 log, and RLU for *L. monocytogenes* ScottA declined by 2 logs over 9 h of stationary phase. A boiling test was further undertaken for the last time point of the cells to investigate the level of ATP loss in stationary phase of *L.*

monocytogenes population. Interestingly, the results show that the intracellular ATP level of boiled cells is nearly the same as the peak value and is higher by 0.5 log of *L. monocytogenes* Fw 03/0035 and 1.5 log of *L. monocytogenes* ScottA, respectively, than unboiled cells.

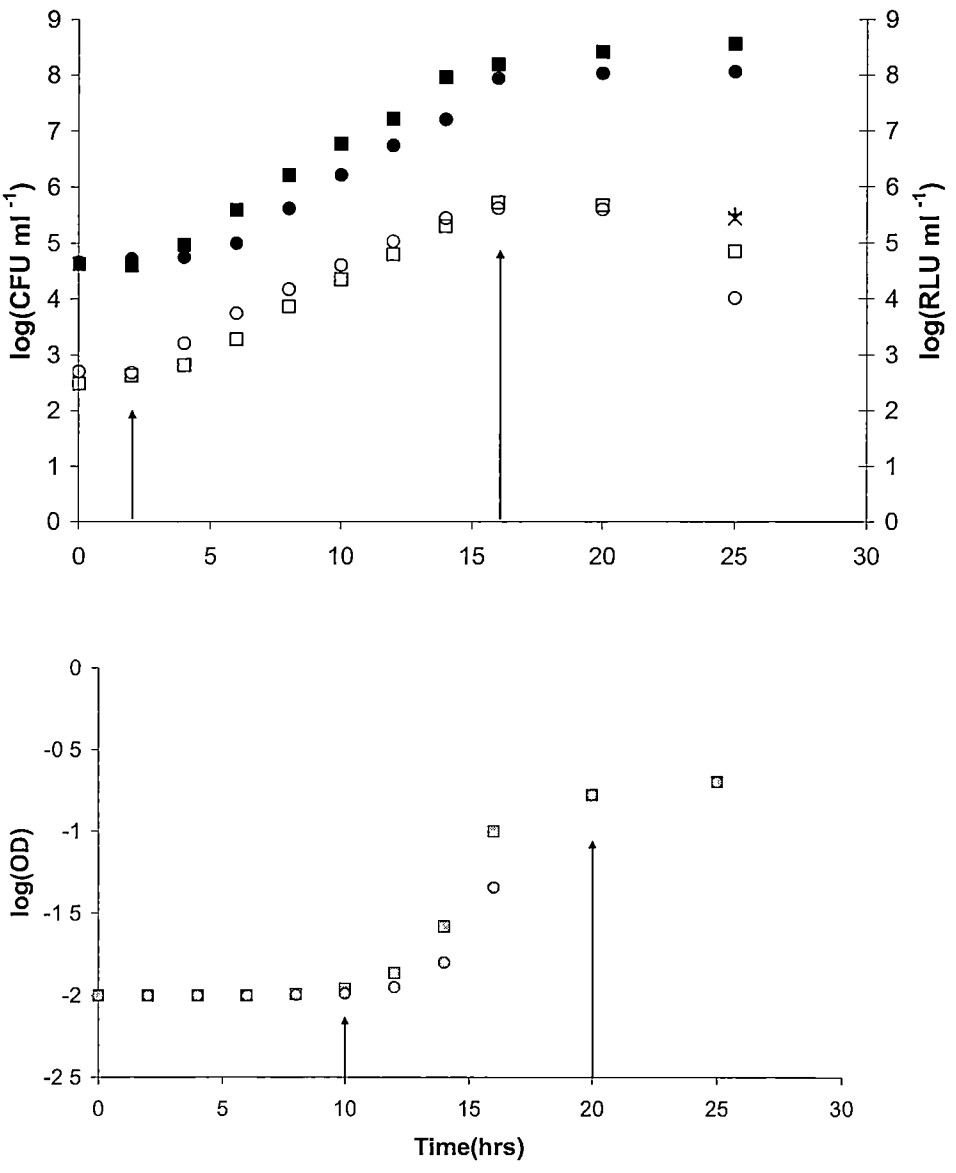


Fig 3.4 *L. monocytogenes* ScottA and Fw 03/0035 were inoculated to TSB-Ye broth and grown to exponential phase at 25°C. Samples were periodically withdrawn for direct comparison of **a**: viable count [*L. monocytogenes* ScottA (•) and Fw 03/0035(■)], ATP method [*L. monocytogenes* ScottA (o) and Fw 03/0035(□)] and **b**:optical density study [*L. monocytogenes* ScottA (◦) and Fw 03/0035(⧈)].For the last sample in **a**, cells were boiled at 100°C for 5 min and followed by ATP method [*L. monocytogenes* ScottA (+) and Fw 03/0035(×)].

However, for optical density (Fig 3.4b), the growth curves appeared to be totally different from viable count and ATP method. OD only registers cell densities over ~10^6 CFU.ml^{-1}(Ullrich et al., 2005). Cells apparently remained longer in lag phase (for 10 h) prior to commencement of exponential phase, and peaked at the different time point on entering stationary phase (i.e. 20 h rather than 16 h). The differences in the critical time points of different phases make the linear growth curve much steeper compared with the viable count and ATP method. Thus, the growth rate for viable count and ATP method are correlated, but are obviously different from the optical density method (see comparison in Table 3.1). The difference between ATP method and viable count are 0.0385 and 0.0278 for strains ScottA and Fw03/0035, respectively, while the averages rate estimates (for both strains) for the two methods are 0.2522 and 0.2285. The difference between the optical density method and the average of those two methods are much larger (0.1210 and 0.0995), while for the OD method itself the estimates are 0.1312 and 0.1290 for *L. monocytogenes* Fw03/0035 and ScottA, respectively. It is clear that growth rates estimated by Optical Density differ significantly to those estimated by ATP/bioluminescence or viable count.

Table 3.1 Growth rate comparisons by three enumeration methods. viable count, ATP and Optical Density Method (time points were selected between two arrows in Fig 3.4).

Methods	*L. moncytogenes* Fw03/0035	average or difference	*L. moncytogenes* ScottA	average or difference
Viable Count	0.2714^a	0.2522^g	0.2146^d	0.2285^j
ATP	0.2329^b	0.0385^h	0.2424^e	0.0278^k
Optical Density	0.1312^c	0.1210^i	0.1290^f	0.0995^l

Note: g=average (a+b); j=average (d+e); h=a-b; k=e-d; i=g-c; l=j-f

3.5 DISCUSSION

As the number of viable microorganisms decreases in the sample during microbial inactivation, the physical, biophysical, and biochemical events associated with those cells will also decrease accordingly, and vice versa. Thus, standard curves correlating parameters such as ATP level (Relative Light Unit) with viable cell counts of the same sample series must be made and then a scattergram must be plotted. Theoretically, the substance, ATP, has a short survival time after cell death and, ideally, any method should detect as low as one viable cell in a sample. Practically, however, the sensitivity of luminometry is usually approximately 10^3 cells ml^{-1}. This technique depends upon the sensitivity offered by the firefly luciferase enzyme and its absolute specificity for ATP which is selectively extracted from the microbial cells, as well as the ability to detect the light emitted. With real samples the sensitivity is often substantially reduced due to quenching of the luciferase by interfering compounds in the sample (Stanley, 1989; Thore et al., 1975). Farkas et al. (2002) consider that present-day commercial reagents and luminometers provide a convenient means of measuring ATP down to about one picogram (10^{-12} g) in a sample volume of 100 µl. This represents the amount of ATP in about one thousand "average" bacterial cells, because the average bacterium contains around 10^{-15} g ATP per cell.

For the application of the ATP method in assessing microbial inactivation, four important assumptions are inherent: (i) all living organisms contain ATP; (ii) ATP is easily extracted from bacterial cells and can be precisely measured, (iii) ATP is not associated with dead cells, and (iv) there exists a fairly constant ratio of ATP to total living cells number.

3.5.1 The Importance of ATP

For the first criterion, ATP is the keystone of all cellular activity; it is a high energy compound in every living cell (Lehninger, 1965); it is an essential ingredient in the initial biochemical steps of substrate utilization in new cell synthesis (Mandelstam and McQuillen, 1968); it is synthesized from intermediate and final reactions of substrate oxidation and is also utilized intracellularly for osmotic and mechanical work (Stanier *et al.*, 1963).

3.5.2 The Key of ATP Extraction

ATP is easily extracted from bacterial cells, however, there may be an incomplete release of nucleotides from the bacteria or losses during subsequent steps, in addition to effects on levels of adenine nucleotides by chemical or enzymic degradation reactions. This may result in an underestimation of ATP, whereas the estimation of the total pool of adenine nucleotides may be decreased. A high yield of ATP is thus the main criterion for a successful extraction. Extraction with TCA yields levels of ATP in bacteria that are either higher than other extraction methods, or very close to the highest yields (Lundin, 1984; Lundin and Thore, 1975a; Lundin and Thore, 1975b; Simpson and Hammond, 1989). However, in those studies, they all used 5% TCA as a standard for ATP extraction from various bacteria species. They did not compare 5% TCA with 10% TCA and did not apply the method to *L. monocytogenes*. In this study, as shown in Fig 3.2, a higher level of TCA, 10%, yielded more ATP from high concentrations of *L. monocytogenes* ScottA and Fw 03/0035. Thus it was selected as the standard extraction method.

3.5.3 The Uniqueness of ATP for Luminescence Reaction

For the fourth criterion, sensitive methods for ATP analysis have been developed. McElroy (1947) reported that luminescence in fireflies has an absolute requirement for ATP and it is the only nucleoside triphosphate that will produce light with purified extract of firefly lantern, which means, no other naturally occurring ribonucleoside triphosphate may substituted for ATP (Hastings, 1968; McElroy, 1947; McElroy and Green, 1956). Patterson *et al.*, (1970) reported that the ATP pool was constant throughout all phases of growth, the variations in cellular ATP content rarely exceed one order of magnitude, and the amount of light produced by firefly lantern extract is directly proportional to the amount of ATP added (Hobson and Summers, 1972). Thus, it would seem that, theoretically, the accuracy and sensitivity of the assay is adequate to enumerate microbial cells.

3.5.4 Is ATP Associated with "Dead" Cells?

However, for the third criterion, as we can see from Fig 3.3a, b and c, when viable cell counts decreased rapidly, the total ATP decreased only gradually. Furthermore, when cells appeared to be "dead" by viable count, there was still a light signal emitted, and which could mean that the cells have no ability to grow on plates, but may still be "alive", but are too damaged to grow. Furthermore, when cells have no ability to grow on the plate, and when the temperature, at which the experiments were undertaken, increased from 25°C, the optimal growth temperature for *L. monocytogenes*, through 35°C, to 45°C, the maximum growth temperature for *L. monocytogenes* (Table 2.1), which is itself lethal to *L. monocytogenes*, the remaining level of the intracellular ATP decreased more rapidly and more

completely, which suggests that the "harsher" the conditions in which the cells were inactivated, the lower the residual intracellular ATP level.

Moreover, when the "dead" cells were boiled (Fig 3.3a), the residual light signal from ATP method was reduced and was then similar to that of the control. This could mean that even when cells are incubated in lethal conditions, there remains an intracellular ATP level that is correlated with their viability rather than their culturability. Thus, in this circumstance, ATP could potentially be used to differentiate cell viability from cell culturability.

Intracellular bacterial ATP varies in a given environment. The cause of the possibility that "dead" cells may contribute to the final ATP measurement, may be related to the extractant chosen to provide maximum recovery of bacterial ATP and minimal inhibition of the luciferase enzyme reaction, the differentiation of bacterial ATP from other sources of ATP if present (Karl, 1980), the sensitivity of the assay, etc. In this study, however, all these potential contributors were precluded, so the only factors left are the environmental conditions known to affect microbial ATP content including growth-preventing pH and water activity.

The manner in which a cell is killed is significant in determining whether ATP will be maintained and therefore measured in the luminescent assays. Agents that rupture the cell, agents that kill without necessarily rupturing, agents that inhibit either cell division or metabolism and death due to ageing in a batch culture can all prevent cell growth or colonies formation, while cells retain some metabolic activity (Chappelle et al., 1977). When sufficient sonication is used as a method of killing

cells they are ruptured. The rate of ATP breakdown was markedly reduced when the ultrasonic treatment was performed at approximately 100°C or at an acidic pH (Chappelle et al., 1977). The same observations have been found by Patterson *et al.*, (1970), i.e. that ATP completely disappeared within two hours after cell death under lethal environmental temperatures, i.e. 103°C.

In the study reported here, multiple hurdles were used to inactivate the cells, including temperature 45°C with pH 3.5 and a_w 0.9. At 45°C, cells were exposed to three lethal hurdles (Table 2.1), as were the cells that were boiled, while at 25 and 35°C, cells were inactivated under non-thermal conditions, which may prevent recovery without necessarily rupturing the cell or causing denaturation of macromolecules. The difference between those mechanisms of injury is potentially highlighted by ATP methods, rupture or denaturation being more likely to be involved in no residual ATP level. Alternatively, treatments that rapidly denature proteins and macro-molecules would be expected to more completely inhibit residual metabolic activity. Thus to interpret the mechanisms of cell injury leading to inactivation, ATP method can be a sensitive tool.

To inhibit cells from growth and metabolism, antibiotics, depending on their specific mode of action are sometimes used in experiments. Nalidixic acid is an antibiotic which inhibits DNA replication in bacteria. Picciolo et al. (1977) reported that in the presence of nalidixic acid there is a continuous decline in viable count until 24 h where no colony forming units can be detected. In contrast, ATP continued to show a small but persistent increase up to 4 h, then a slight decrease, but still remain measurable in the culture for up to 24 h. The antibiotic did not cause

the loss of the intracellular ATP pool, although the cells were rendered non-dividable, i.e. "non-culturalbe".

For an aging bacterial population, Chappelle et al. (1977) found that when a culture of *E. coli* was grown over an extended time period, i.e., 15 days, and the stationary phase extended into the death phase, that viable cell numbers decrease rapidly, while intracellular ATP levels diminish only gradually. Thus, in situations where cell death is the result of nutrient limitations or end-product inhibition, the intracellular ATP levels do not undergo the drastic changes seen in other types of cell death, such as rupturing. However, the phenomenon is different when cells enter stationary phase (Fig 3.4); intracellular ATP levels decreased rapidly compared with viable cells numbers which remained relatively unchanged, and for boiled cells intracellular ATP level were higher than untreated samples, which will be further discussed in Section 3.5.6.

The question arises whether only live cells were measured in the work described in this chapter. In the case of cells that did not receive a lethal temperature treatment it is likely that the total number of non-ruptured organism was measured (Fig 3.3 a and b), some of which were injured, or dying and not able to form colony forming units, but still retaining some biosynthetic activity explaining why the kinetics of these two measurements are dramatically different. Whether these particular organisms would be counted if the measurement of respiration or metabolism were made or if some attempt were made to restore their ability to divide on different agar nutrient medium is worthy of further investigation.

Due to the consistency with the basic physiology of organisms, it is reasonable that cells do not immediately lose all their ATP concomitant with the loss of their ability to divide. Thus the concept of viable organisms from colony forming units needs to be distinguished. In most practical aspects, they are equivalent. It is possible, however, that some viable cells do not form colonies in the cases mentioned above. Therefore, a portion of the total ATP from such non-ruptured cells, in this study of 25°C and 35°C, may originate from the "dead" (i.e., either nonviable, injured or debilitated) cells.

Another question that arises is whether ATP is detected only in viable cells. In an actively metabolizing cell, the rate of ATP breakdown (decrease in steady-state concentration) is the net result of enzymic synthesis and hydrolysis, and chemical hydrolysis is the only mechanism for ATP destruction. The half-life of ATP may vary from a few minutes to several hours, depending upon such factors as pH, temperature (Deustach and Johnson, 1968; Hobson and Summers, 1972). In a stressed cell, the reduction of the ATP pool might reflect an increased energy demand resulting from the efforts to maintain homeostasis and to relieve the burden of osmotic stress on compatible solute transportation. Thus, again the difference on how long the ATP would last, or the contribution of ATP from "nonliving" cells, would depend upon the specific cause of death or cell damage, as well as the chemical and physical nature of the environment.

This complex process of cell damage influenced by time, temperature, and environmental conditions is named "injury" (Sallam and Donnelly, 1992; Smith and Marmer, 1991). Postgate (1969) reported microorganisms that survive an inimical

process may be injured, either metabolically (non-ruptured cells), structurally (ruptured cells) or both, rather than killed by sublethal levels of stressors. As discussed above, the ATP method and viable count seems equivalent for ruptured cells, but may assess totally different properties for non-ruptured cells. It is obvious that the "recovery" of a non-ruptured cell is easier than a ruptured one. Thus the viability of a cell depends on the ability of the cell to recover from injury. Returning to the question of whether ATP only can be found in viable cells, the answer appears to depend, in part, on whether an environment is provided for such cells to recover on agar plates.

3.5.5 Further Investigation on Microbial Growth

Due to the variable correlation between ATP content and viable count of microbial cells subject to inactivation by non-thermal lethal conditions, a growth study was undertaken to assess the reliability of ATP assays as an enumeration method (Fig 3.4). The ATP content, assessed by luminometry of *L. monocytogenes* ScottA and Fw03/0035 increased in parallel with their viable cell counts in the exponential phase of the growth. Thus, viable count is related to the amount of ATP in growing cells. Similarly, Hanberger *et al.*, (1995) reported that there was a good correlation between bioluminescence and viable counts in growing cultures of *E. coli*, and this is also consistent with previous studies including species other than *E. coli* (Hanberger et al., 1993; Molin et al., 1983; Thore et al., 1975). Moreover, several authors have found a linear correlation exists between ATP content and viable plate count, and that the ATP method gave a more valid cell count in some cases for some species than plating because of clumping characteristics (Deustach and Johnson, 1968; Lundin and Thore, 1975b; Stannard and Wood, 1983). Thus, as a

rapid method, ATP measurement can be used to assess microbial growth and may apply to all microbial species.

3.5.6 Stationary Phase Investigation

At the commencement of the stationary phase, however, the light output of *L. monocytogenes* ScottA and Fw03/0035 dropped significantly (Fig 3.4), and a similar phenomenon was reported by Eaton *et al.* (1993) for cultures of a bioluminescent transformant of *L. lactis*. In that study, the light emission characteristically reached a peak just before the culture entered stationary phase and thereafter decreased. A possible explanation for this phenomenon may be that the decrease of luminometric activity might indicate the transformation of the cells into a metabolically less active stationary state as a stress-adaptive response to nutrient depletion, "metabolic crowding", or, even inimical processes. It seems that as cells remain in stationary phase, the ATP levels decrease (Chappelle et al., 1977), This might explain why the boiled cells' intracellular ATP level is higher than not boiled (Fig 3.4a), because although the samples were removed at the same time, it takes hours for unboiled samples to be assayed while they were stored in 4°C, compared with the boiling cells which were immediately moved into a 100°C water bath, thus, denatured hydrolytic enzymes that could catabolize ATP.

On the other hand, the difference of the bioluminescence activities between growing and stationary-phase cells may limit the utility of luminometry as an alternative method for viable count estimation for situations when the physiological status of the population is unknown. However, as pointed out by Stewart (1990), changing luminometric activity of bioluminescent cells in parallel with changes in their

metabolic intensity may be utilized to follow, sensitively, changes in their physiological and metabolic status, and as a response to environmental stresses. Similarly, measurement of ATP in this study (Fig 3.3), always produced a sudden dramatic drop corresponding with the environmental stresses and shock at the commencement of the inactivation experiments.

3.5.7 Another Point of View

All of the above discussion relates to the comparison of viable count and ATP measurements. However, there is a broader consideration, and inherently assesses whether the viable count is the most reliable method for bacterial enumeration. From the preceding discussion, it is conceivable that another method might be superior to the viable count in this respect. Limiting consideration of such a method only for its ability to predict colony counts, could limit its potential application to produce other useful information about the system being studied (Fung, 2000).

3.6 CONCLUSION

The aim of this study was to investigate a more rapid and sensitive method to study microbial inactivation. However, the results show that the inactivation rates and kinetics were not comparable between the ATP method and viable count. In contrast, for the growth study, the growth rates, estimated by either method are strongly correlated. Thus, it can be concluded from this study that luminometry can be applied (after its calibration to viable cell counts) in growth studies as a labour and material saving, selective, data capture method for quick estimation of the size of viable populations by assessing ATP levels, but only when the target cells are in the actively growing physiological state. It also can be used to sensitively detect physiological and metabolic status changes in cell populations, such as from exponential to stationary phase or responding to environmental stresses and inimical processes. Another application of ATP assays might be a specific assessment of cells viability when they were ruptured in lethal stresses. In conclusion, the major advantage of the luminescence ATP procedure is to differentiate non-culturable cells from non-viable cells and its rapidity and sensitivity compared to the standard or conventional plate count procedure.

CHAPTER 4

ELONGATION FACTOR EF-TU AS AN INDICATOR OF CELL VIABILITY

4.1 ABSTRACT

To enhance understanding of the processes leading to inactivation of food-borne pathogens in inimical food environments, the efficacy of quantitative PCR (QPCR) in the analysis of cell viability and activity of *L. monocytogenes* was assessed. The expression of the *tuf* gene which codes for the protein elongation factor EF-Tu was measured. This gene is highly expressed under normal growth conditions and is essential for transcription and translation. Cultures of *L. monocytogenes* ScottA and Fw 03/0035 were held in TSB-Ye poised at a_w 0.90 and pH 3.50 at 25°C and samples were periodically withdrawn for targeted *tuf* gene QPCR and viable counts. Although viable cell numbers decreased from 10^8 to less than the detection level (1.3×10^1 cells ml^{-1}), the *tuf* mRNA level remained stable. To determine whether *tuf* mRNA was synthesised despite the apparent lack of viable cells, or whether levels of this transcript were more stable than anticipated, additional experiments were undertaken. Cells were held under: (i) mildly lethal temperature (55°C), (ii) presence of rifampin (which inhibits DNA-dependent RNA polymerase) and (iii) high temperature and rifampin. Rifampin reduced *tuf* gene expression much more completely than inimical pH and water activity; mildly lethal temperature also resulted in a rapid loss of viability, but retained higher *tuf* gene levels. This raises questions about whether *L. monocytogenes* under lethal conditions of pH and a_w or mildly lethal high temperature retain viability despite being non-culturable.

4.2 INTRODUCTION

Conventional culture-based methods for microbial enumeration are labour intensive and time consuming, in many cases requiring days to complete. To overcome these limitations, in recent years many molecular biology based approaches for the rapid detection of *L. monocytogenes* have been developed, including immunoassays, nucleic acid-based hybridization assays, and PCR- based methods (Fung, 2000). Although these techniques have been somewhat successful at decreasing the time necessary for pathogen detection, they may not be able to demonstrate whether the cells are alive or dead. PCR-gene probe methods are very specific and can be extremely sensitive, but since DNA can survive treatments that kill the cell (Josephson et al., 1993), they do not distinguish between living and dead organisms. An alternative method for detection of bacterial cells that combines sensitivity and specificity with the ability to differentiate between viable and nonviable cells is needed.

In contrast to DNA, messenger RNA (mRNA) is turned over rapidly in living bacterial cells, with most mRNA species having a half-life from 40 s to 20 min (Kushner, 1996; Yaron and Matthews, 2002). Detection of mRNA rather than DNA might therefore be a better indicator of viability, and still retain the advantages of specificity and sensitivity. Using the enzyme reverse transcriptase (from retroviruses) the DNA associated with a mRNA can be amplified by PCR (Sheridan et al., 1998). By choosing a mRNA that codes for a protein that is produced constitutively, and amplifying it, one has an assay of cell viability. Reverse transcriptase PCR (RT-PCR) can also be made 'real-time' and quantitative

(Sheridan et al., 1999). Thus, it might also provide a rapid means of assessing cell viability and number and be able to measure rates of inactivation more rapidly and easily than conventional viable plate counts.

The *tuf* gene was selected for this study because it encodes for the protein synthesis elongation factor Tu (EF-Tu), which is highly expressed under normal growth conditions and is essential for transcription and translation (Bosch et al., 1983). EF-Tu is the most abundant protein in *E. coli* and is encoded by two genes, *tufA* and *tufB* (Van der Meide et al., 1983). In contrast, there is only one *tuf* gene in *L. monocytogenes* (Bosch et al., 1983). The importance of EF-Tu is in its GTP-bound (active) form, EF-Tu binds aminoacylated tRNAs to form the so-called ternary complex. At the decoding site of the ribosome, the ternary complex is "tested" for a codon-anticodon match; only if the proper aminoacyl-tRNA has been found, can translation then be performed properly (Van der Meide et al., 1983).

From the results presented in Chapter 3, it was found that ATP, assessed using a luminometry procedure, could still be detected even in "dead" cells. Similar results were reported by Sheridan, et al (1998) for a one-tube RT-PCR method for detecting mRNA from the *tufA* gene of *E. coli.* They reported that mRNA was detected immediately after the cells had been killed by heat or ethanol but gradually disappeared with time when dead cells were held at room temperature. In heat-killed cells, some mRNA targets became undetectable after 2 to 16 h, whereas after ethanol treatment, mRNA was still detected after 16 h. Thus, in this chapter the question investigated is whether cells held in non-growth environments are still

"alive", i.e. actively maintaining their structure and function, or whether they are metabolically inert when subjected to certain inimical conditions.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial Strains, Media, Reagents, Solutions and Equipment

Details of bacterial strains, bacteriological media, chemical reagents, solutions and equipment (including software), together with the methods for bacterial maintenance and recovery, are given in Appendix A. All experiments described in this chapter employed *L. monocytogenes* ScottA and *L. monocytogenes* Fw03/0035.

4.3.2 "RNA" based Rapid Enumeration method

4.3.2.1 Extraction of RNA: comparison of protocols

Four protocols for extraction of mRNA from bacterial cultures were assessed as part of this study and are described below. Cell concentration in the samples was determined by viable count as described in Section 2.3.2.5.

Protocol 1 Mechanical Disruption

0.5ml aliquots of samples (stationary phase populations of *L. monocytogenes*, Section 2.3.2.2) were removed to a 2ml "Safe-Lock" tube, which contained 25-50mg acid-washed glass beads (150-600μm diameter) and 1ml RNAprotect™ Bacteria Reagent, and were mixed immediately by vortexing for 5 s. Tubes were then incubated for 5 min at room temperature (RT) and then centrifuged for 10 min

at 5000×*g*. The supernatant was removed by pipetting and 350µl of Buffer RLT (RNeasy® Mini Kit) and 3.5µl β-mercaptoethanol were added and the sample then vortexed vigorously for 5-10 s. Disrupted cells were placed in a bead-beater for 10 s at 2655×*g* and this was repeated three times. Cells were centrifuged for 10 s at maximum speed (~21000×g). The supernatant was transferred to a new eppendolf tube, and an equal volume of ethanol (70%) was added and mixed well.

Protocol 2 Enzymatic Lysis and Mechanical Disruption

0.5ml aliquots of samples (stationary phase populations of *L. monocytogenes*, Section 2.3.2.2) were removed to a 2ml Safe-Lock tube, which contained 1ml RNAprotect™ Bacteria Reagent and were mixed immediately by vortexing for 5 s. Tubes were then incubated for 5 min at RT, and then centrifuged for 10 min at 5000×*g*. The supernatant was removed by pipetting and 100µl of TE buffer (10mM Tris·Cl, 1 mM EDTA, pH 8.0) containing 15 mg ml^{-1} lysozyme was added and mixed by vortexing for 10 s. Tubes were incubated at 25°C for 16 h. 350µl of Buffer RLT (RNeasy® Mini Kit) and 3.5µl β-mercaptoethanol were added and mixed by vortexing vigorously for 5-10 s. 25-50mg of acid-washed glass beads (150-600µm diameter) were added into the pellet. Cells were disrupted with a bead-beater at 2655×*g* for 10 s three times, and then centrifuged for 10 s at maximum speed (~21000×*g*). The supernatant was transferred by pipetting to a new tube, and 220µl of ethanol (96-100%) was added to the supernatant and mixed well by pipetting.

Protocol 3 Enzymatic Lysis, Proteinase K Digestion, and Mechanical Disruption

0.5ml aliquots of samples (stationary phase populations of *L. monocytogenes*, Section 2.3.2.2) were removed to a 2ml Safe-Lock tube, which contained 1ml RNAprotect™ Bacteria Reagent and were mixed immediately by vortexing for 5 s. Tubes were then incubated for 5 min at RT, and then centrifuged for 10 min at 5000×g. The supernatant was removed by pipetting and 100µl of TE buffer (10mM Tris·Cl, 1 mM EDTA, pH 8.0) containing 15 mg ml^{-1} lysozyme and 20µl QIAGEN Proteinase K was added and mixed by vortexing for 10 s. Tubes were incubated at 25°C for 16 h. 700µl of Buffer RLT (RNeasy® Mini Kit) and 7µl β-mercaptoethanol were added and mixed by vortexing vigorously for 5-10 s. 25-50mg of acid-washed glass beads (150-600µm diameter) were added into the pellet. Cells were disrupted with a bead-beater at 2655 *g* for 10 s three times, and then centrifuged for 10 s at maximum speed (~21000×*g*). The supernatant was transferred by pipetting to a new tube, and 590µl of ethanol (80%) was added to the supernatant and mixed well by pipetting.

Protocol 4 Enzymatic Lysis, Proteinase K Digestion

0.5ml aliquots of samples (stationary phase populations of *L. monocytogenes*, Section 2.3.2.2) were removed to a 2ml Safe-Lock tube, which contained 1ml RNAprotect™ Bacteria Reagent and were mixed immediately by vortexing for 5 s. Tubes were then incubated for 5 min at RT, and then centrifuged for 10 min at 5000×g. The supernatant was removed by pipetting and 100µl of TE buffer (10mM Tris·Cl, 1 mM EDTA, pH 8.0) containing 15 mg ml^{-1} lysozyme and 20µl QIAGEN

Proteinase K was added. The pellet was carefully resuspended by pipetting up and down several times and mixed by vortexing for 10 s. Tubes were incubated at 25°C for 16 h. 700µl of Buffer RLT (RNeasy® Mini Kit) and 7µl β-mercaptoethanol were added and mixed by vortexing vigorously for 5-10 s. Tubes were then centrifuged for 10 s at maximum speed (~21000×*g*). The supernatant was transferred by pipetting to a new tube, and 590µl of ethanol (80%) was added to the supernatant and mixed well by pipetting.

4.3.2.2 Purification of Total RNA from *L. monocytogenes* Lysate Using the RNeasy Mini Kit with DNA-free Step

All lysate including any precipitate that may have formed was transferred to a RNeasy® Mini spin column in a 2ml tube and centrifuged for 15 s at 8000×g. The flow-through was then discarded. 700µl Buffer RW1 (RNeasy® Mini Kit) was added to the RNeasy® Mini spin column, and centrifuged for 15 s at 8000×g to wash the spin column membrane, and the flow-through discarded (and the collection tube reused). 10µl DNase I stock solution was added to 70µl Buffer RDD and mixed gently by inverting the tube. 80µl of the DNase I incubation mix was pipetted directly onto the spin-column membrane, and placed on the bench top for 15 min. 350 µl buffer RW1 was pipetted into the spin column, and centrifuged for 15 s at 8000×g. The RNeasy® Mini spin column was placed in a new 2ml collection tube. 500µl Buffer RPE (RNeasy® Mini Kit) was added to the RNeasy® Mini spin column, and centrifuged for 15 s at 8000×g to wash the spin column membrane. This step was repeated and another 500µl Buffer RPE added, and centrifuged for 2 min at 8000×g. The RNeasy® Mini spin column was placed in a new 1.5 ml

collection tube. 30µl of RNase-free water was added directly to the spin column membrane, left for 5 min to settle and then centrifuged for 1 min at 8000×g to elute the RNA. This step was then repeated and the collected RNA was stored at -80°C until ready for further processing.

4.3.2.3 Checking Total RNA Concentration

To select a suitable protocol for RNA extraction, the total amount of RNA extracted was measured using the *Pico*fluor™ Fluorometer. It was always calibrated before performing any sample analysis using a blank [10µl aliquot of Quant-iT™ RiboGreen® (RNA Assay Kit) in 1990µl of TE buffer] and an RNA standard (100ng μl^{-1}). Samples were compared with the calibrated standard to determine RNA concentration in ng. μl^{-1}.

4.3.2.4 Testing the Efficiency of the RNA Extraction by Serial Dilution

Stationary phase population of *L. monocytogenes* (Section 2.3.2.2) were serially diluted in TSB-ye broth. For each dilution, 0.5ml aliquots of samples were subjected to RNA extraction protocol 3 (Section 4.3.2.1), and then followed by a purification step (Section 4.3.2.2). For samples with a concentration of $<10^3$ cells per ml, 50ml of each dilution were centrifuged for 10 min at 5000×*g*, and the pelleted cells resuspended in 1ml of RNAprotect™ Bacteria Reagent. 10µl aliquots were dispensed into a tube with 100µl of TE buffer (10mM Tris·Cl, 1 mM EDTA, pH 8.0) containing 15 mg ml^{-1} lysozyme and 20µl QIAGEN Proteinase K. RNA

extraction protocol 3 was undertaken, followed by a purification step (Section 4.3.2.2).

4.3.2.5 QPCR Assay

Primer design

Primers for *tufA* were designed using information from the following three websites, and are detailed in Table 4.1.

1. "Genelist", at the Institute Pasteur, France: http://genolist.pasteur.fr/ListiList/;
2. "Primer 3", hosted by the Whitehead Institute at the Massachusetts, Institute of Technology, USA: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi;
3. GenScript Corporation Website, USA: https://www.genscript.com/ssl-bin/app/primer (real-time specific primer design site).

Table 4.1 Gene Works primers for *tufA* gene

Gene	Forward primer (5'-3')	Reverse primer (3'-5')
tufA	AGG TGA AGC TGA CTG GGA AG	CTG GCA TCA TGA ATG GTT TG

Running Real-Time Assay and Standard Curve Construction

A 2µl aliquot of each unknown RNA sample was added to 18µl of reaction mix (Table 4.2), which was kept on ice prior to placing in a thermocycler. Thermal cycling conditions were as follows: 30 min at 50°C, 15 min at 95°C followed by 40 repeats of 15 s at 94°C, 30 s at 50°C and 30 s at 72°C. Data collection was performed by software (Rotor gene^{TM} 6000) according to the instructions of the

manufacturer. In each run two negative controls (RNA free water) were included. For each RNA sample analysed, measurements of gene expression were replicated three times, and the mean of these values was plotted as log (CFU) (Section 2.3.2.5) against log (RNA concentration) to construct the standard curve.

Table 4.2 Quantitative PCR Reaction Mix

Reaction components	1x(µl)
QuantiTect SYBR Green RT-PCR Master Mix	10
QuantiTect RT Mix	0.2
F Primer	1
R Primer	1
RNA free water	5.8

4.3.2.6 Relative Gene Expression

The number of copies of cDNA ml^{-1}, as a measure of the amount of mRNA (*tuf* gene copies) obtained by the QPCR assay, was divided by the number of bacteria ml^{-1} in the sample. The number of *tuf* gene copies was divided by the number of culturable bacteria at the time point at which inactivation was commenced i.e. by adding acid (pH 3.50) and salt (a_w 0.900) (Section 2.3.2.3). This quotient (number of cDNA copies CFU^{-1} or number of *tuf* gene copies $cell^{-1}$) represents the amount of RNA expressed per bacterium in the sample.

4.3.2.7 Enumeration of Intracellular mRNA and Construction of Inactivation Curves of *L. monocytogenes*

Immediately prior to the inimical treatment (pH 3.50 and a_w 0.900, Section 2.3.2.3 and 2.3.2.4), and either at regular intervals throughout or at a specific time point as

described, 1ml aliquots were removed. 0.5ml of the sample was immediately used for RNA extraction using protocol 3 and the remaining sample was using for viable count. All RNA extracted samples were then subjected to the purification step, followed by real-time PCR. For each RNA sample, gene expression was measured three times, and the mean of these values was plotted as log (number of *tuf* gene copies $cell^{-1}$) against log (CFU) (Section 2.3.2.5).

4.3.3 Exponential Growth, pH, a_w and Heat Shock, and Rifampin Challenge

Exponential phase populations of *L. monocytogenes* were prepared (see Section 3.3.4.1). Populations were exposed to inimical treatment of pH 3.50 and a_w 0.900 as described in Section 2.3.2.3 and 2.3.2.4, and mildly lethal heat stress was applied by placing the cultures in a water-bath pre-set at 55°C. To study the effect of rifampin, rifampin was added to an exponential phase culture to a final concentration of 5mg ml^{-1}. All experiments were carried out at least in duplicate.

4.3.4 Parallel Evaluation of QPCR Method of Assessing *L. monocytogenes* Inactivation with Viable Count

The viability of each population was estimated by two methods in parallel: a culture-based enumeration method and a mRNA based enumeration method. Immediately prior to the inimical treatment (Section 4.3.3), and either at regular intervals throughout, or at a specific time point as described, two 1ml aliquots were removed from populations being assessed. 100µl from one of the aliquots was used

for viable count estimation by the plate count method (Section 2.3.2.5). The other 0.5ml aliquots were used for assay of *tufA* mRNA expression (Section 4.3.2). Survival curves were constructed by plotting log of the CFU.ml^{-1} and log (*tuf* gene copies number cell^{-1}) against time.

4.4 RESULTS

4.4.1 The Selection of Extraction Protocols

Of the four protocols which were used for RNA extraction (described in Section 4.3.2.1), protocol 3 yielded the highest amount of RNA (783.4 ng µl^{-1}) compared with the yield from the other three protocols of 72.9, 210.5 and 194.1 ng µl^{-1}, respectively. Thus, protocol 3 was selected for subsequent experiments.

Table 4.3 Yield of the different extraction protocols for RNA as assessed using the *Pico*fluor™ Fluorometer

Protocols	RNA yield (ng µl^{-1})
Protocol 1	72 9
Protocol 2	210.5
Protocol 3	783.4
Protocol 4	194.1

4.4.2 Testing RNA Extraction Efficiency

The efficiency of RNA extraction using protocol 3 (Section 4.3.2.3) is shown in Fig 4.1. Stationary phase cells of *L. monocytogenes* ScottA were serially diluted

from10^8 CFU ml^{-1}. The RNA concentration of each sample was measured by two methods: QPCR of *tufA* cDNA and fluorometry. The data (Fig 4.1) shows the limit of sensitivity of the QPCR assay was 10^3 CFU ml^{-1}, which are 4 logs (10^7 CFU ml^{-1}) more sensitive than to the sensitivity limit of the fluorometry method.

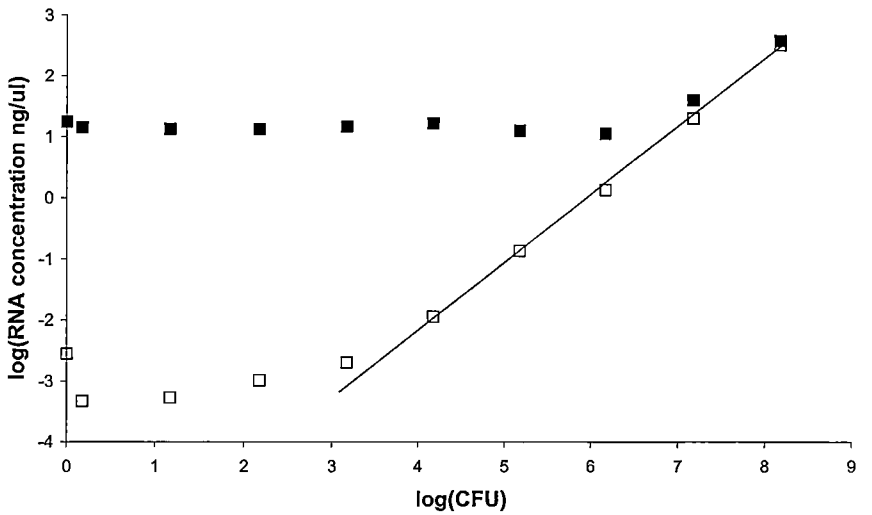


Fig 4.1 Stationary phase *L. monocytogenes ScottA* cells were ten-fold serially diluted and RNA was extracted from these samples. The RNA concentrations were measured and reported as log [total RNA by *Pico*fluor™ Fluorometer (■)] and log [*tuf* RNA by QPCR assay (□)].

4.4.3 Salt and Acid Challenge

Exponential phase cultures of *L. monocytogenes* ScottA and Fw 03/0035 were inactivated by exposing cells to a_w 0.90 and pH 3.50 at 25°C. The results (Fig 4.2) showed that viable cell numbers decreased from 10^7 to less than the detection level (1.3×10^1 cells ml^{-1}), while the *tuf* gene mRNA copy number remained stable. The level of *tuf* gene copies per cell was based on the number of cells at time 0 and was not further adjusted for the apparent decrease in cell numbers as assessed by viable count. In other words, the total cell number is assumed to remain at a constant level

despite the reduction in viable cell count. Of particular interest is that mRNA signals can still be detected even when the cells have no ability to produce colonies on the agar plates, suggesting that cells may still be "alive", but have suffered injuries preventing growth. On the other hand, the sensitivity limit of the QPCR method is 10^3 CFU ml^{-1}, below this limit, false results may be obtained, if it is assumed that non viable cells are "dead" and do not contribute to the total mRNA signal (Fig 4.1).

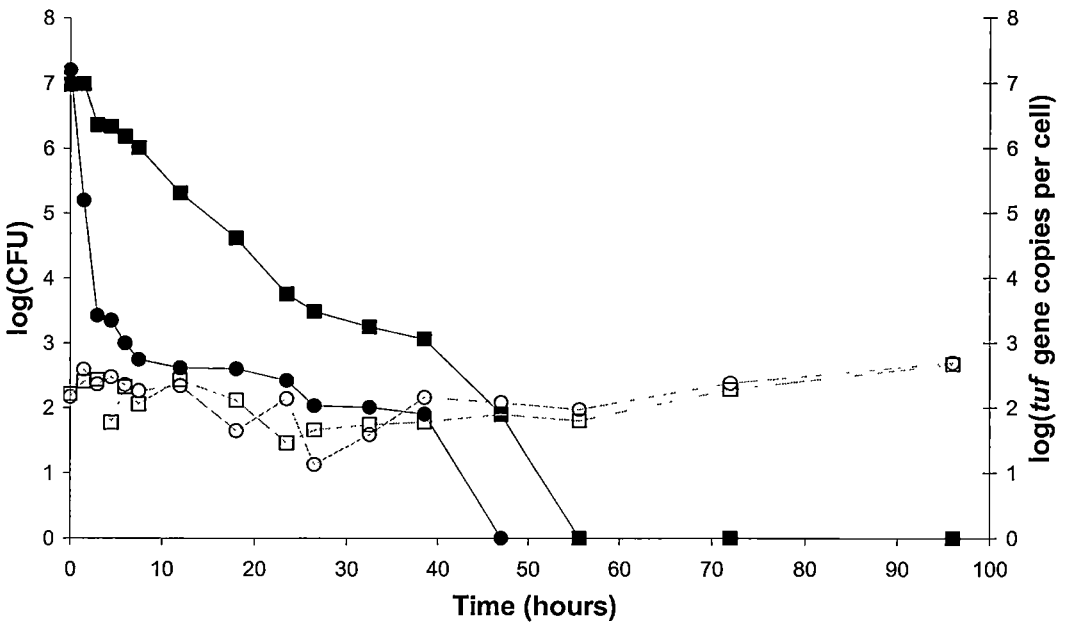
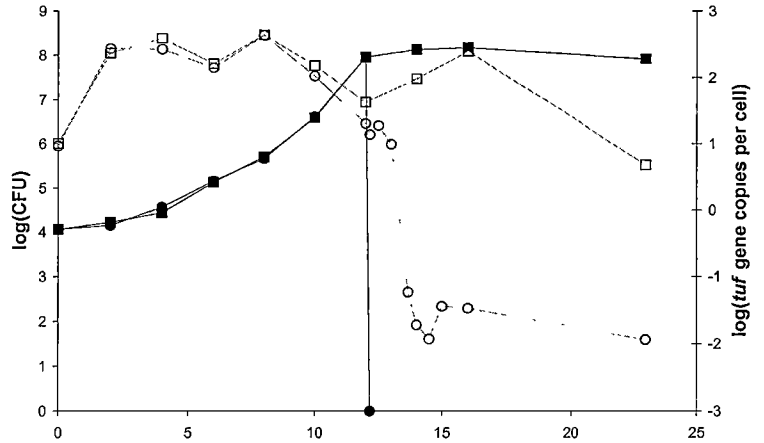


Fig 4.2 Exponential phase populations of *L. monocytogenes* ScottA and Fw03/0035 were held in TSB-Ye broth at temperature 25°C, and the broth was adjusted by addition of HCl and NaCl to pH 3.50 and a_w 0.90, which prevents cell growth. QPCR and viable count methods were employed to enumerate *L. monocytogenes* Fw 03/0035 and ScottA. Viable count Fw 03/0035(■) and ScottA (●), QPCR *tuf* gene copies Fw 03/0035(□) and ScottA (o).

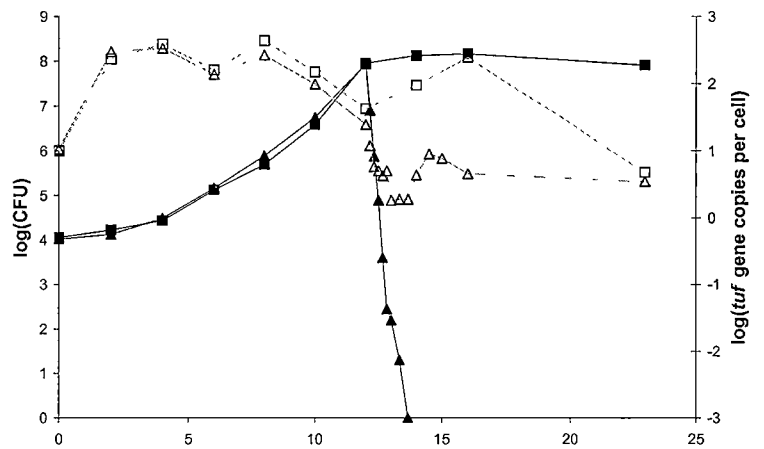
4.4.4 Antibiotic and High Temperature Challenge

To explain the phenomenon reported above, i.e. that RNA did not break down as expected despite that viable count declined rapidly, further investigations were

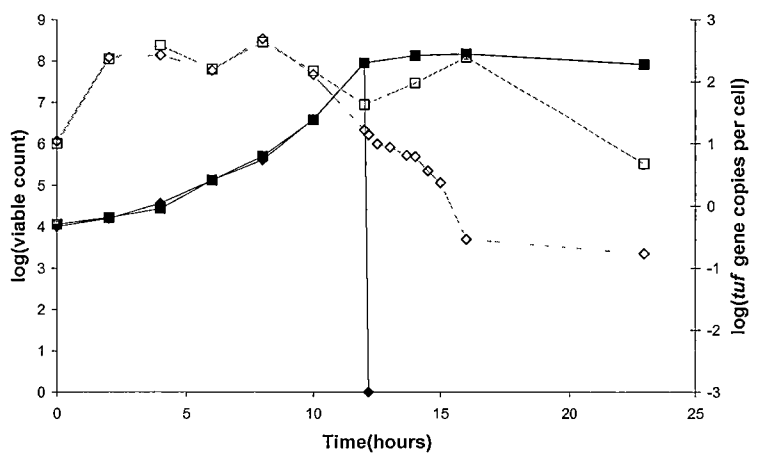
undertaken. Antibiotic, high temperature (55°C) and combined antibiotic plus high temperature (55°C) challenges were imposed on exponential phase cultures of *L. monocytogenes* ScottA. The results are presented in Fig 4.3 a-c. Cells were inoculated at 10^4 CFU ml^{-1} and allowed to grow exponentially achieving 10^8 CFU ml^{-1} when they commenced stationary phase. In the exponential phase, cells quickly built up their *tuf* gene pool from 10 to nearly 1000 copies $cell^{-1}$, but dropped to less than 10 copies $cell^{-1}$ again near the commencement of stationary phase. Note that, in this case, the estimate of "*tuf* gene copies per cell" is based on the viable count estimated at each sampling time until the time point of commencing the treatments, beyond which it was assumed that there is no further changes on the total cell numbers despite the reduction in viable count. For antibiotic (rifampin) treatment alone (Fig 4.3a), immediately after the treatment, the cells became "non-viable" by viable count, while for the QPCR assay the *tuf* gene pool decreased dramatically from nearly 20 copies $cell^{-1}$ to 1 copy in 100 cells. For the 55°C treatment alone (Fig 4.3b), there were no colonies culturable on plates after one hour exposure, but for QPCR assay the *tuf* gene pool remained stable in a range of 5 to 15 copies $cell^{-1}$ 10 hr after the treatment. A combination of antibiotic and 55°C treatment (Fig 4.3c) is similar to antibiotic alone: cells became inactivated immediately after the treatment, while QPCR assay results indicated the *tuf* gene pool gradually decreased from around 20 copies $cell^{-1}$ to 1 copy in 10 cells.



a



b



c

Fig 4.3 a-c In all Figures, *tuf* gene expression of *L. monocytogenes ScottA* was measured by the QPCR method compared with viable count. Three challenges are
a: antibiotic (rifampin), QPCR method (o) *vs* viable count (•)
b: high temperature (55°C), QPCR method (Δ) *vs* viable count (▲)
c: antibiotic and high temperature, QPCR method (◇) *vs* viable count (◆).
In all Figures, the blank control, QPCR method (□) *vs* viable count (■).

In Table 4.4, inactivation rates of viable count and mRNA were calculated by using the Microsoft® Excel trendline function and starting from the time point at which the inimical stresses were applied. For the viable count data, the inactivation rate for the rifampin and rifampin+55°C treatments were similar, but for 55°C treatment inactivation is an order of magnitude slower. When applying the QPCR method, the inactivation rates for 55°C challenge was only 0.02. However, for rifampin and rifampin+55°C treatments the inactivation rates were 0.29 and 0.19, respectively. Thus, between the different treatments inactivation rates were proportionally similar, however inactivate rates based on QPCR and viable count methods differed by approximately 200-fold.

Table 4.4 The comparison of inactivation rates of the three challenges including antibiotic (rifampin), mildly lethal temperature (55°C) and both combined by two enumeration methods: QPCR method and viable count.

Challenges	Inactivation rates	
	QPCR method log(*tuf* gene copies. $cell^{-1}$. h)	Viable count log(CFU.ml^{-1}.h)
Antibiotic (rifampin)	0.29	46.84
Mildly lethal temperature (55°C)	0 02	4.80
Rifampin + 55°C	0 19	46.84

4.5 DISCUSSION

It is hard to define the terms "life" or "living" from "death" because there is no unique property common to all living things (Pirie, 1937). Microbiologists tend to define "live" organisms as viable organisms, i.e. those that can multiply to form colonies on agar plates or cause visible turbidity in broth, but there are exceptions: the so-called "viable but nonculturable" organisms (Roszak and Colwell, 1987) and those that can multiply in liquid media but only to low maximum cell densities (Button et al., 1993). For practical purposes, a "live" bacterial cell is a viable bacterial cell and is the one that has the potential to multiply under suitable conditions (Barer et al., 1998; Bloomfield et al., 1998).

4.5.1 Alternative Enumeration Methods

Sheridan et al. (1998) proposed that a useful indicator of viability should have three properties: that it is present only in viable cells; that the kinetics of its disappearance is related to loss of viability, and that it disappears from cells soon after death.

Due to the presence of the 2'-hydroxyl group of ribose, the phosphodiester bonds of RNA are more susceptible to hydrolysis than those of DNA, particularly in the presence of divalent cations (Kushner, 1996). RNA is therefore more labile than DNA and more susceptible to degradation caused directly by deleterious treatments, such as heating or acidification (Sheridan et al., 1998). Sheridan et al. (1999) found that DNA can survive even when cells are killed by harsh treatments such as autoclaving, but mRNA is more susceptible than DNA to degradation and was not detected in autoclaved or boiled cells. Also, Bej et al. (1996) detected no mRNA in

cells of *Vibrio cholerae* that were inactivated by heat or starvation. Similarly, Patel et al. (1993) did not detect heat shock protein mRNA from *Mycobacterium leprae* killed by heat treatment.

In this work, a QPCR method was developed for quantifying a specific mRNA, the *tufA* gene from *L. monocytogenes* ScottA. Levels of specific mRNAs detected by RT-PCR vary depending on the physiological state of cells, and in certain cases, levels can be 10000-fold greater in exponential-phase than in stationary-phase cells (Holmstrom et al., 1999). Thus, to maximise sensitivity of the experiments, exponential phase cells were chosen for inactivation. The results from Fig 4.2 shows that the mRNA remained stable in cells inactivated by salt (a_w 0.90) and acid (pH 3.50), and that it persisted in cells that are "dead" by standard methods of evaluation, which means they have no ability to grow on non-selective media plates. However, the results obtained in this study are different from the studies cited above.

There can be two explanations of the contradictory findings. The first is that the cells are truly dead, but that the extent of mRNA degradation varies according to the type and severity of treatments, i. e. mRNA may survive longer in some circumstances. It is observed that in cells inactivated by milder treatments, the correlation is not absolute and in some cases the mRNA can persist for several hours (Coutard et al., 2005; Yaron and Matthews, 2002). The length of time that mRNA persists will depend on both the method by which cells were killed and the post-mortem holding conditions (Sheridan et al., 1999). In this study, the data shows that mRNA can persist for at least 10 h, but further work is required to

characterize the decay rates of mRNA in dead cells under a range of conditions before the limitations of the method are fully defined. Also, because cells have to be killed before their nucleic acid can be extracted, there can be no absolute correlation between the presence of mRNA and viability (Kushner, 1996). Therefore mRNA may not be an absolute indicator of viability according to criteria given above.

The other explanation would be that bacteria are capable of responding to environmental stresses by employing various survival mechanisms, and one of them is entering the viable but nonculturable (VBNC) state (Roszak and Colwell, 1987). Cells existing in this state retain viability and the potential for infection (Colwell et al., 1996) but they are no longer culturable on "routine" laboratory media. To date, it has been reported that 60 bacterial species, including pathogens, non-pathogens, Gram-negative and Gram-positive microorganisms, are capable of entering and persisting within such a survival state (Oliver, 2005). Cells have been reported to enter this state in response to natural stresses such as starvation; unfavourable temperatures, osmotic levels, or oxygen concentrations; and exposure to harmful light (Oliver, 2000). The VBNC state is characterized by cell dwarfing and decreases in macromolecular synthesis, nutrient transport, and respiration rates (Porter et al., 1995). In addition, several reports have indicated the continued transcription of genes by cells in the VBNC state in the laboratory (Coutard et al., 2005). In this work, when the cells appear to be "dead" on plates (Fig 4.2), they might have entered a VBNC state instead, thus the mRNA signal may be a real reflection of the cells viability.

The microscopic appearance of the cells was investigated in this study as well, such as using BacLight™ live/dead techniques, however, the method did not produce meaningful results. By using BacLight, live and dead cells should fluoresce green and red, respectively, under UV microscopy. The problem was that the colours faded too quickly to capture the exact count of green or red cells. Thus, the results aren't included in the thesis. However, further method development of the BacLight™ live/dead techniques would add great value to assess the potential for VBNC state of cells in future work.

4.5.2 Exploration of Non-thermal Inactivation Mechanisms

In living cells, most mRNA turns over rapidly, reflecting a balance between the synthesis of mRNA and its degradation by RNases (Alifano et al., 1994; Belasoco, 1993). In "dead" cells, mRNA synthesis (if any) is likely to be slower and nuclease activity will continue to degrade any mRNA present. Presumably, mRNA would disappear most rapidly from cells killed by treatments that do not inactivate the degradative RNase enzymes. Conversely, mRNA may remain intact for longer in cells killed by treatments that also inactivate RNase or render the RNA resistant to attack (Kushner, 1996).

To understand whether *tuf* mRNA was synthesised despite the apparent lack of viable cells or whether levels of this transcript were more stable than anticipated, further experiments were undertaken as shown in Fig 4.3. Detectable mRNA signal decreased more quickly and to a lower level from antibiotic-inactivated cells than from heat-inactivated cells. The antibiotic used, rifampin, inhibits the initiation of

chain formation in the process of RNA synthesis and inhibits bacterial transcription by interfering with normal RNA polymerase activity (Hobby and Lenert, 1968). Consequently, the different levels of mRNA signal after treatments must be related to the different effects of heat and rifampin on the process of mRNA breakdown and/or inhibition of mRNA synthesis in inactivated cells. The relatively rapid disappearance of mRNA from antibiotic inactivated cells means that when cell mRNA synthesis is prevented that mRNA degradation is rapid. The *tuf* gene transcript becomes undetected. Conversely, the relatively high levels of mRNA observed in heat inactivated cells suggest that *tuf* gene was synthesised despite apparent lack of viable cells, because if mRNA persists after the treatment, there should be no such difference (2 logs) on the copy numbers between antibiotic inactivated cells and heat inactivated cells. These results suggest that the answer to the question in the first experiment is that the stable mRNA signal from acid and salt inactivated cells is due to cells maintaining their structure and function, including synthesis of mRNA, but appearing to enter a VBNC-like state.

Sheridan (1998) demonstrated that of the four species of nucleic acid, mRNA is the most promising candidate as an indicator of viability in bacteria. Similarly, Klein and Juneja (1997) showed a good correlation between the presence of mRNA and the viability of *L. monocytogenes* when comparing growing cells with those killed by autoclaving. However, in this study, when cells inactivated by milder treatments, such as acid, salt and/or heat, the correlation is not absolute, because the cells appear to be "dead" by viable count, but actually appear to be still "alive" in maintaining their structure or function by synthesising mRNA.

Using the definition described at the beginning of this Discussion, it can be difficult to define the term "living" and, when that applies to microorganisms, the simple answer is by determining the viable count. In this study, the data shows that viable count estimated by colony formation on agar culture plates is not a reliable indicator when cells are exposed to mildly lethal conditions, because the cells appear to enter a VBNC state. In this way, QPCR methods based on mRNA detection, may be still be useful for particular applications as they provide a better indication of the presence of viable cells in food, clinical, or environmental samples.

Kell et al. (1998) reported that cells may become nonculturable when they are damaged in an essential cellular component that may lead them to lose the ability to divide. However, there is little information on what the essential cellular component is and what the minimum or threshold concentrations of the components are to instruct the cells not to replicate *in vitro*. Thus, further investigation is needed to explore the possible cellular components that relates to cell culturability, as opposed to cellular maintenance and repair.

4.6 CONCLUSION

The purpose of this study was i) to investigate an alternative way of determining viability through measurement of the abundance of the mRNA coding elongation factor EF-Tu and ii) better understand mechanisms of non-thermal inactivation. The results show that inactivation with pH3.5/a_w0.9 resulted in a more than 10^8 decline in viable cells, however, total *tuf* gene expression stayed relatively stable. The expression of *tuf* was reduced much more completely in the presence of rifampin with a three log reduction compared with mildly lethal temperature in which only a half log reduction was observed. Thus, *L. monocytogenes* under mildly lethal conditions of pH and a_w or high temperature may retain possible cellular activity and thus viability, despite being rendered non-culturable.

CHAPTER 5

MECHANISMS OF NON-THERMAL INACTIVATION

-A TIME COURSE STUDY

5.1 ABSTRACT

Low pH and low a_w are characteristics of certain foods such as fermented meat or cheese and are also as parts of "multiple hurdles" to prevent the growth and survival of contaminating pathogens, such as *L. monocytogenes*. Genomic microarray analysis was performed to determine the simultaneous effects of low pH (3.5) and low a_w (0.90) on exponential phase *L. monocytogenes* ScottA cells, in a time-course experiment (5 min, 24 h, 48 h , 72 h). Gene set enrichment analysis indicated that increased expression of genes associated with branched chain amino acid biosynthesis, glycine/serine/threonine metabolism, pantothenate/CoA biosynthesis and peptidases and histidine metabolism occurs. On the other hand, suppression of expression related to flagellar assembly, type III secretion, chemotaxis, and fructose/mannose metabolism associated genes was suggested. Genes that maintained relatively strong expression under simultaneous low pH and low a_w code L-lactate dehydrogenase (lmo0210), single-stranded DNA-binding protein (lmo0045) and non-heme iron-binding ferritin (lmo0943), respectively. Other strongly expressed genes code proteins involved in general stress responses, and included uncharacterised transporters.

5.2 INTRODUCTION

The concept of microarrays was first proposed in 1980s (Bains and Smith, 1988; Drmanac et al., 1989; Khrapko et al., 1989; Southern et al., 1992). By the 1990s, the concept was developed to involve either DNA fragments or synthetic oligonucleotides arrayed on various substrates, including nylon membranes, plastic and glass (Lockhart et al., 1996; Schena et al., 1995). Currently, microarrays are described as microchips, biochips, DNA chips, or gene chips and have emerged as a widely accepted functional genomics technology for large-scale genomic analysis. In particular, DNA arrays have been used to monitor mRNA abundance levels of differentially expressed genes under different cell growth conditions or in response to environmental stresses (Lockhart et al., 1996; Schena et al., 1996; DeRisi et al., 1997; Wodicka et al., 1997; Ye et al., 2000; Thompson et al., 2002; Liu et al., 2003)

Microarray experiments generate massive data sets, which must be analyzed and interpreted in a rapid and meaningful way, thus microarray experiments involve several stages. Firstly, to improve the efficiency and reliability of experimental data and to make the data analysis and interpretation as simple and powerful as possible, careful experimental design is needed (Yang and Speed, 2002). It can be classified into the three categories as shown in Fig 5.1: reference design, all-pairs design, and loop design. i) In the reference design scheme, all treatment samples are labeled with one dye and are hybridized, respectively, with a common reference sample labeled with another dye. This indirect design is used widely in gene expression studies and it is especially suitable when cells are cultured under different physiological conditions. Since it is straightforward, the reference design is a widely used approach for identifying gene expression patterns associated with

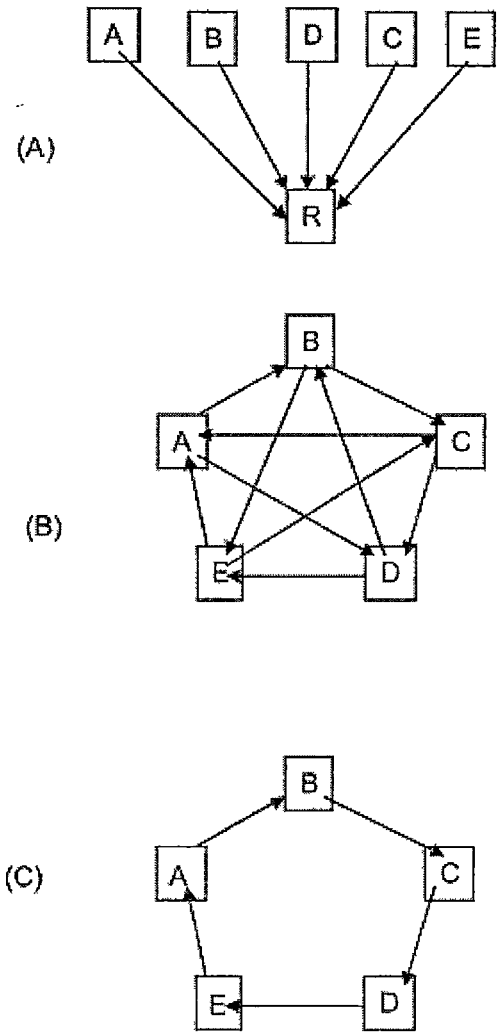


Fig 5.1 Illustrations of basic types of microarray experimental design schemes with five treatment samples. (A) Reference design. The five treatment samples (A–E) are labeled with one dye and hybridized, respectively, with the common reference sample R, which is labeled with the other dye. Altogether five hybridizations are needed. (B) All-pair design. Each sample is labeled twice with red and twice with green. Ten pair-wise hybridizations are needed. (C) Loop design. Each sample is labeled once with red and once with green. Five successive pair hybridizations are needed (after Ehrenreich, 2006).

various physiological states (DeRisi et al., 1997; Ye et al., 2000; Tao et al., 1999).

ii) In the all-pairs design scheme, all of the treatment samples are labeled with different fluorescent dyes and directly hybridized. Cells are grown under a specific physiological condition and then harvested at different time points during growth

(DeRisi et al., 1997; Liu et al., 2003). The main advantage of this design is that more precise comparisons among different treatment samples can be obtained. iii) In the loop design, all of the treatments are successively connected as a loop (Kerr and Churchill, 2001). The advantage of loop design is that it requires far fewer slides than the all-pairs design. However, long paths between some pairs of treatment samples are needed in larger loops, and thus some comparisons are much less precise than others (Yang and Speed, 2002).

Secondly, the basic approach to microarray-based gene expression studies is outlined in Fig 5.2. In a typical microarray experiment for monitoring gene expression, gene-specific PCR primers are designed based on whole-genome sequence information and synthesized. Gene-specific fragments are then amplified with specific primers, purified, and arrayed on solid substrates (Ehrenreich, 2006). Once the microarrays are ready, total cellular RNA isolated from bacterial cells grown under two different conditions (a control and experimental condition) is amplified into cDNA via the enzyme, reverse transcriptase (Dharmadi and Gonzalez, 2004) and fluorescently labeled with different dyes (Cy3 or Cy5). The microarray is then simultaneously hybridized with the fluorescently tagged cDNA from the test and reference samples. The signal intensity of each fluorescent dye on the array is then measured with a confocal laser scanning microscope or CCD camera (Yang and Speed, 2002). The quantitative ratio of red (Cy5) to green (Cy3) signal for each spot reflects the relative abundance of that particular gene in the two experimental samples (Wildsmith and Elcock, 2001). With appropriate controls, the intensity can be converted into biologically relevant outputs (e.g., differential gene expression ratio) and the data can be analyzed with various statistical methods (Southern,

2000).

Thirdly, statistical considerations are frequently to the fore in the analysis of microarray data (Xia et al., 2005). The acquired red/green ratios must be normalized to adjust for dye-bias and for any systematic variation other than that due to the differences between the RNA samples being studied. As researchers sift through massive amounts of data, the normalized ratios are analyzed by various graphical and numerical means to find differentially expressed (DE) genes (Dharmadi and Gonzalez, 2004).

In the refereed literature, there are several papers that utlised DNA microarray analysis to study responses of *L.monocytogenes* or Gram positive bacterial species exposed to selected stress conditions, or using mutant strains to study various alternative σ -factors or regulators at the transcriptome level. Raengpradub et al., (2008) studied alternative sigma factor σ^B which is activated following exposure to a number of environmental stress conditions. They found out that σ^B regulon includes more than 140 genes which are important in stress responses, transcriptional regulation, carbohydrate metabolism, and transport and σ^B also affects motility and chemotaxis.

Arous et al. (2004) investigated σ^{54}, encoded by the *rpoN* gene. The results indicated that σ^{54} is mainly involved in the control of carbohydrate metabolism *via* direct regulation of PTS activity, alternation of the pyruvate pool and modulation of carbon catabolite regulation. Bennett et al. (2007) analyzed the CodY family of

global regulatory proteins by transcriptome analyses, and demonstrated that the genes involved are responsive to both GTP and branched chain amino acids. PrfA was investigated by Milohanic et al. (2003) suggesting that it may directly or indirectly activate different sets of genes in association with different sigma factors in *L. monocytogenes*.

Following from Chapter 4, the main research question addressed in the studies described here is again whether cells in non-growth environments are inactivated but maintain their structures or whether they remain metabolically active but non-culturable. From Chapter 4, it seems that metabolically active cells loss their ability to grow and form colonies. A further question is that when cells are suddenly exposed to a stressful environment, such as one that is hyper-osmotic and acidic, what genetic responses are associated with the shift from a living cell with the ability to divide, move, respire and perform complex chemical reactions to a cell that apparently loses the ability to from colonies on plates.

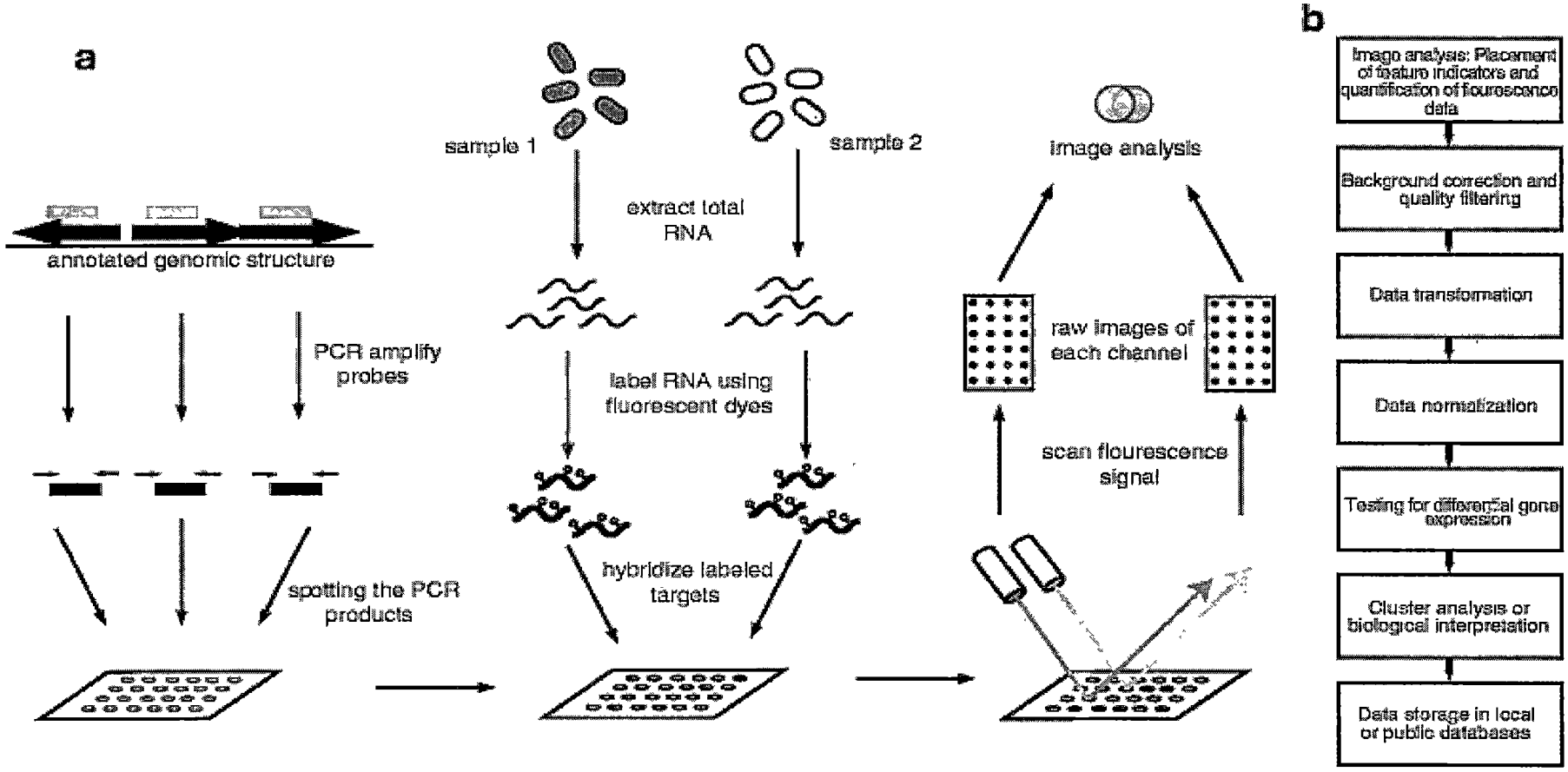


Fig 5.2 Main steps in transcription analysis with microarrays. a Probes are generated from an annotated genome sequence and spotted on a microarray slide. For target preparation, RNA is extracted from two experimental conditions and labeled with fluorescent dyes by reverse transcription. The labeled target is then hybridized with the array, and the fluorescence of the features is determined using an array scanner. b After image analysis, quality filtering, data transformation, and normalization are done. The remaining steps are dependent on the experiment, but in most cases, the data are tested for different gene expression, clustered, and finally stored in a database for further analysis. Reproduced from (Ehrenreich, 2006).

5.3 MATERIALS AND METHODS

5.3.1 Bacterial Strains, Media, Reagents, and Equipment

Details of bacterial strains, bacteriological media, chemical reagents, and equipment (including software), together with the methods for bacterial maintenance and recovery, are given in Appendix A. All experiments described in this chapter employed *L. monocytogenes* ScottA.

5.3.2 Preparation of Log Phase Inactivation for a Large Volume (1 L)

To yield the required amount RNA for microarray experiments, exponential phase populations of *L. monocytogenes* ScottA were prepared in a large volume (1 L), by transferring five colonies from BHAP to 100 ml TSB-Ye in a 250 ml Erlenmeyer flask and incubating statically at 37°C for 24 h, to achieve a population density of approximately 9.0 log CFU.mL^{-1}. The populations were diluted 10^{-7} in 100 ml TSB-Ye in a 250 ml Erlenmeyer flask and incubated at 25°C until just turbid (typically 30 h), which correlated to a population density of approximately 7.0 log CFU.mL^{-1}. At this time, populations were diluted 1/10 000 in 1000 ml TSB-Ye and incubated at 25°C in a water bath with shaking at 60 or 100 oscillations per minute until just turbid (typically 10 h) providing a population density of approximately 7.0 log CFU.mL^{-1}. Immediately following inoculation and at the end of the treatment, a 2 ml aliquot was withdrawn for a_w and pH measurements.

Inactivation Trial 1

UV treated NaCl (15% to provide a_w 0.90) and 4.6 ml of 32% concentrated HCl (to

achieve pH 3.50) was directly added to the cell suspension indicated above (Section 5.3.2)

Inactivation Trial 2

Based on the Inactivation Trial 1, the shaking speed of the water bath after inimical treatment (pH 3.5 and a_w 0.90) was increased from 60 to 100 oscillations per minute.

Inactivation Trial 3

500 ml TSB-Ye broth with high salt (30%) and acid (4.6 ml of 32% concentrated HCl) were prepared before hand, and were gradually poured into 500 ml of culture of *L. monocytogenes* (Section 5.3.2) to create a final pH of 3.50 and a_w of 0.90.

Inactivation Trial 4

Exponential phase cells (Section 5.3.2) were pelleted by centrifugation at 1964 *g* for 15 min at room temperature (RT) in a Universal 16A centrifuge. The supernatant was removed by pipetting and the cells were resuspended in a 1ml aliquot of a a_w (0.900) and pH (3.50) broth before being resuspended in 1L of the same broth.

Inactivation Trial 5

After 17h, once an hour for 6 h, six of 50 ml aliquots of cells were pelleted by centrifugation at 1964 *g* for 15 min at RT in a Universal 16A centrifuge. The supernatant was removed by pipetting and the cells were resuspended in 1ml aliquot of the a_w (0.900) and pH (3.50) broth, and cultured in a further 49 ml of the same broth.

Inactivation Trial 6

20×50 ml aliquots of cells were pelleted by centrifugation at 1964 *g* for 15 min at RT in a Universal 16A centrifuge. The supernatant was removed by pipetting, the cells resuspended in 1ml of a_w (0.900) and pH (3.50) broth and were transferred to 20×50ml of a_w (0.900) and pH (3.50) broth.

5.3.3 RNA Isolation

5.3.3.1 RNA Extraction

25ml aliquots of untreated exponential phase samples and 50 ml of stressed samples from Inactivation Trial 6 were removed and centrifuged at 1964 *g* for 15 min. The supernatant was decanted and 3ml of culture media was used to resuspend the cell pellet by flicking the bottom of the tube; 6 ml (2 volumes) of RNAprotect™ Bacteria Reagent (RNAprotect™ Bacteria reagent) was then pipetted into the tubes. The tubes were mixed immediately by vortexing for 5 sec and incubated for 5 min at room temperature (15-25°C). RNA stabilized biomass was harvested by centrifugation for 10 min at 5000×g. The supernatant was removed by pipetting, and 1ml of TE buffer (10mM Tris·Cl, 1 mM EDTA, pH 8.0) containing 15 mg/ml lysozyme and 20µl QIAGEN Proteinase K was added and mixed by vortexing for 10 sec. The suspension was incubated at 25°C over night, 4ml of Buffer RLT (RNeasy® Midi Kit) and 40µl β-mercaptoethanol were added and then vortexed vigorously for 5-10 sec. 25-50mg acid-washed glass beads (150-600µm diameter) were added and cells disrupted using a bead-beater (3 cycles, 3000 rpm for 20 sec), and then centrifuged for 10 sec at maximum speed. The supernatant was transferred (4ml) by pipetting to a new tube, to which was added 2.8ml of ethanol (100%) with

vigorous shaking.

5.3.3.2 RNA Purification

The lysate (supernatant), including any precipitate that may have formed, was transferred to an RNeasy Midi spin column in a 15ml tube, and centrifuged for 5 min at 5000×g. The flow-through was discarded. Four ml Buffer RW1 (RNeasy® Midi Kit) was then added to the RNeasy Midi spin column, and centrifuged for 5 min at 5000×g to wash the spin column membrane (discarding the flow-through) and reusing the collection tube. 4ml buffer RW1 was pipetted into the spin column, and the columns centrifuged for 5 min at 5000×g to wash the column again. The flow through was discarded and 2.5 ml of Buffer RPE (RNeasy® Midi Kit) was then added to the spin column, and centrifuged for 5 min at 5000×g. This step was repeated. The spin column was placed with a new 15 ml collection tube and centrifuged for another 5 min to dry the silica-gel membrane. The spin column was transferred to a new 15ml collection tube to elute the RNA sample by pipetting 100μl of the RNase-free water directly onto the spin column membrane. The tube was allowed to stand for 5 min before centrifugation for 5 min at 5000×g to elute the RNA. The elution step was repeated with the first eluate to obtain a higher total RNA concentration.

5.3.3.3 Formaldehyde Agarose Gel Electrophoresis

Formaldehyde-agarose (FA) gel electrophoresis was used to assure the quality of the RNA samples. FA at 1.2% (w/v) was prepared by mixing 0.24g agarose and 18ml 1×FA gel buffer (200 mM 3-[N-morpholino]propanesulfonic acid, 50 mM sodium

acetate, 10 mM EDTA, pH to 7.0 with NaOH). The mixture was heated to melt the agarose and 1.8ml of 37% (v/v) formaldehyde and 1 µl of a 10 mg ml^{-1} ethidium bromide stock solution were added to the mixture before the gel was poured into, and allowed to set onto, the gel support.

The RNA samples for FA gel electrophoresis was prepared as follow: 2 µl of 5×loading buffer (16 µL saturated bromophenol blue solution, 80 µL 500 mM EDTA, pH 8.0, 720 µL 37% formaldehyde, 2 mL 100% glycerol, 3084 µL formamide, 4 mL 10×FA buffer, add RNase-free water to 10 mL) was added to 8 µl of RNA sample. The RNA-loading buffer sample was incubated for 5 min at 65°C and then chilled on ice prior to being loaded onto the FA gel. The gel was run at 100V for 40 min. The gel was visualized on an UV transilluminator.

5.3.4 Microarray analysis (Australian Genomic Research Facility Ltd)

L. monocytogenes ScottA RNA samples were hybridized to a custom *L. monocytogenes* strain EGD-e microarray. For each of the treatments, 3 biological replicate RNA samples were used in the analysis. The array included 2857 × 70 bp oligonucleotides (Operon Technologies, Huntsville, AL, USA), representing all predicted protein coding genes and pseudogenes of the complete, published genome of *L. monocytogenes* EGD-e (GenBank accession no. AL591824; (Glaser et al., 2001). Microarray set up and analysis followed the methods of Bowman et al. (2008). In short, oligonucleotides were arrayed onto glass slides using quill pens at the Australian Genomic Research Facility Ltd. (Walter & Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia,) with each spot possessing a12 µm

diameter. RNA was converted to cDNA and postlabelled with Cy5 (red) and Cy3 (green) fluorescent dyes using the SuperScript Indirect cDNA Labelling System (Invitrogen). Slides were pre-hybdridised in a hybrisation solution (25% vol./vol. formamide, 5 × SSC buffer – 0.75 mol l^{-1} NaCl, 0.075 mol l^{-1} trisodium citrate, pH 7.0, 0.1% sodium dodecyl sulfate) containing 10 mg ml^{-1} bovine serum albumin for 45 min at 42°C. Slides were then rinsed twice in distilled water and air-dryed. Hybridisation was performed in a humid hybridisation chamber at 42°C for 16-20 h using a hybridisation solution containing 0.42 μg μl^{-1} human Cot1 DNA, 0.62 μg μl^{-1} polyA and 0.83 μg μl^{-1} salmon sperm DNA. Slides were washed at room temperature in 1 × SSC buffer containing 0.2% SDS for 5 min and again in 0.1 × SSC buffer containing 0.2% SDS for 5 min. The slides were then further washed twice in 0.1 × SSC buffer at room temperature for 2 min. Slides were then dried and subsequently scanned using a GenePix 4000A scanner (Axon Instruments). Downstream processing used the GenePix-Pro software package to generate gpx files from TIFF array images.

5.3.5 Gene set enrichment analysis

5.3.5.1 Normalization

For each RNA sample, there are two data sets for technical replication, while for each time point of inactivation duplicate RNA samples were prepared. Therefore, for each time point, there are four data sets. Normalization of raw data and subsequent statistical testing was performed with the WebArray Online platform http://bioinformatics.skcc.org/webarray/ (Xia et al., 2005). Within-array normalization used the global LOESS procedure. Between each array quantile

normalization was used to ensure intensities had the same empirical distribution across arrays and across channels. The significance of differential expression was analysed using linear modal statistical analysis (Smyth, 2004). Spacings LOESS histogram analyses (using WebArray) (Storey et al., 2005) were used to estimate the conditional false discovery rate (FDR), the expected proportion of false positives conditioned on having k 'significant' findings (Pounds and Cheng, 2004). A significant alteration in expression was designated as a 2-fold or greater change.

5.3.5.2 Predicted or known function and functional category

Gene designations, predicted functions and functional categorisation of coded proteins from the *L. monocytogenes* EDG-e genome was based on information obtained from the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.ad.jp/kegg/), ListiList (http://genolist.pasteur.fr/ListiList/), and DAVID (http://david.abcc.ncifcrf.gov) bioinformatic databases. Major functional categories (equivalent to Clustered Orthologous Gene classes) and subcategories are defined in Bowman et al. (2008).

5.3.5.3 Calculation

A *t*-test based procedure as described by Boorsma et al. (2005) was utilised to score the changes in expression of predefined sets of genes and methods were given as follows: for a given gene group G, the *t*-value is given by the following formula:

$$t_G = \frac{\mu_G - \mu_{G'}}{s\sqrt{\frac{1}{N_G} + \frac{1}{N_{G'}}}},$$

where

$$s = \sqrt{\frac{(N_G - 1) \times s^2_G + (N_{G'} - 1) \times s^2_{G'}}{N_G + N_{G'} - 2}}.$$

Here μ_G is the mean expression log-ratio of the N_G genes in gene group G, $\mu_{G'}$ is the mean expression log-ratio of the remaining $N_{G'}$ genes and s is the pooled standard deviation, as obtained from the estimated variances for groups G and G'. The associated two-tailed p-value (TDIST function in Microsoft Excel) can be calculated from t using the t-distribution with $N_G - 2$ degrees of freedom In addition to functional categories, the *T-value* scores for gene sets (regulons) under direct or indirect control of transcriptional regulators SigB (Raengpradub et al., 2008) PrfA (Milohanic et al., 2003), CodY (Bennett et al., 2007) and RpoN (Arous et al., 2004) were also determined.

5.4 RESULTS

5.4.1 The problems with large volume (1L) inactivation

When exponential phase cells were inactivated by inimical pH and water activity in a 1L volume (required to obtain a sufficient RNA yield for the microarray study), various problems were encountered as shown in Fig 5.3 a to d. For inactivation trial 1 (Fig 5.3a) there were no culturable cells detected immediately after the treatment and cell clumps were formed at the bottom of the 2 L flask. To better control the cell inactivation, water bath shaking was increased from 60 to 100 oscillations per minute, but the same phenomena happened, as shown in Fig 5.3 b. It is possible that the direct addition of concentrated acid and salt to the culture might cause extremely rapid inactivation. To assess this, the culture was initially grown in 500ml

broths, and acid and salt in another 500ml broth was gradually added to the culture. However, as Fig 5.3c shows, the results were the same. To further explore this phenomenon, the harvest step (Section 2.3.2.4), centrifugation and resuspension of log phase cells before inoculation to the stress broth, was employed. However, the result was the same (Fig 5.3d) with cells losing culturability almost immediately.

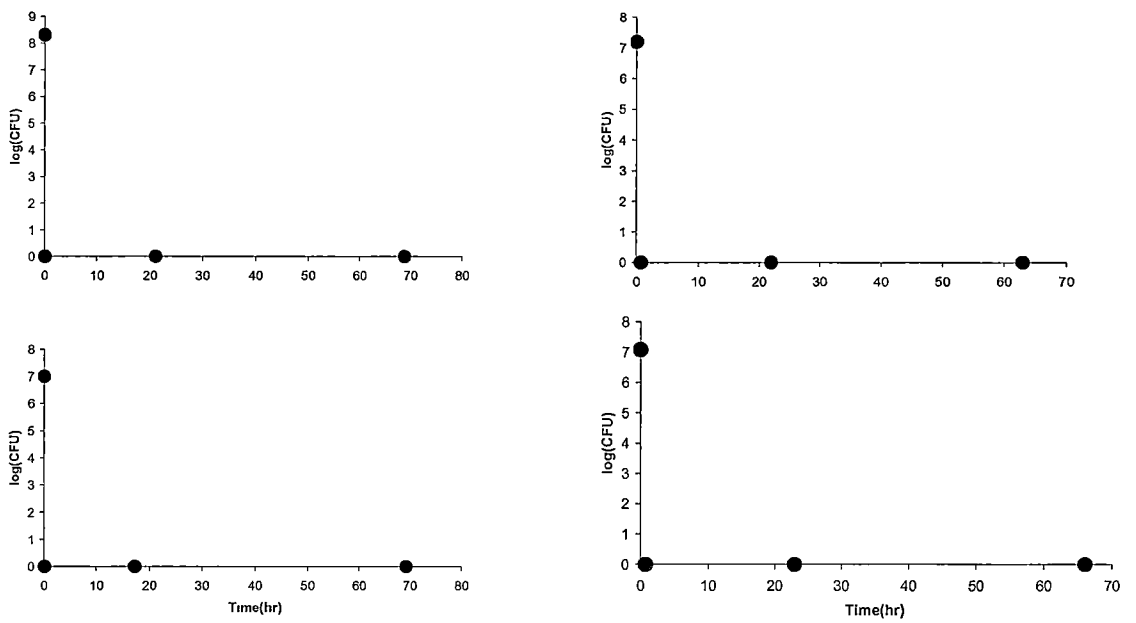


Fig 5.3 a-d Instantaneous loss of culturability (Inactivation Trial 1-4) The inactivation of *L. monocytogenes* ScottA by high salt (a_w 0.9) and acid (pH 3.5) concentration (•) in a large volume 1L

5.4.2 The reuse of small volume (50ml) inactivation

In inactivation trial 5, a smaller culture volume was used to resolve this problem with samples taken at different cell densities as indicated in Fig 5.4. In this case it was observed that it took longer for cells to lose their culturabilty than when higher cell densities were used. When cells were at $10^{6\,57\text{-}6\,9}$ cell ml^{-1}, it took >20h for them

to become undetectable in 0.1ml; when they were at $10^{7\text{-}72}$ cell ml^{-1}, it took ~50h and when they are at $10^{>8}$ cell ml^{-1}, it took >80h.

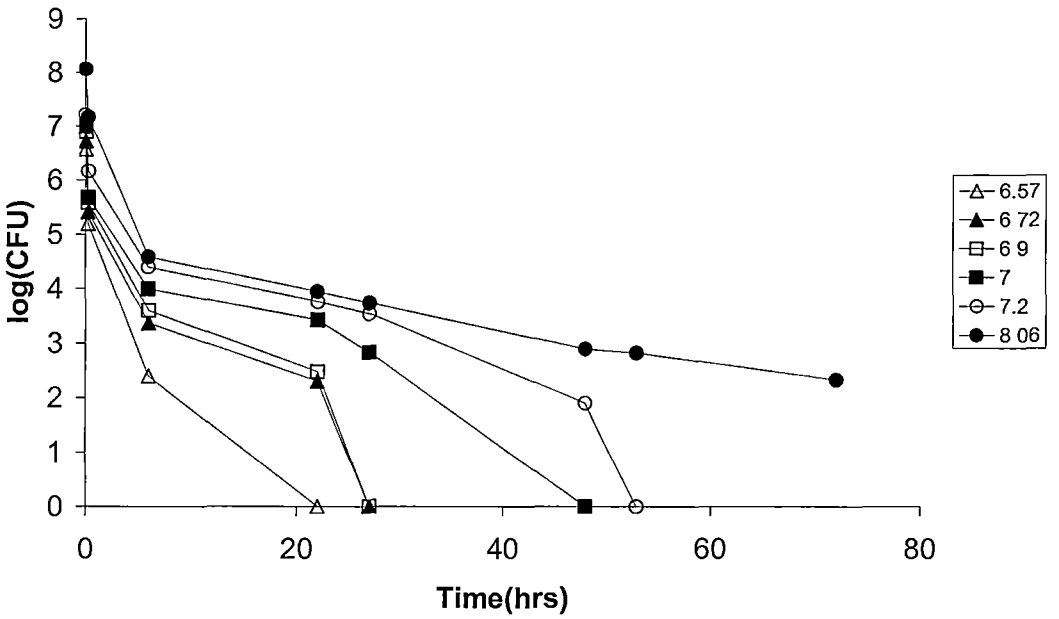


Fig 5.4 The inactivation of *L. monocytogenes* ScottA by high salt (a_w 0.9) and acid (pH 3.5) concentration in a small volume (50ml) and sampling at different cell density: 6.57 (Δ) 6 72(▲) 6.9(□) 7(■) 7.2(○) 8.06 (●)

Thus, an alternate approach was used where several small volumes (50ml of cells) were inactivated individually to make up a final volume 1L. The results are shown in Figs 5.4 and 5.5 for cell culturability. It demonstrates slower inactivation in the experiment, which suggests that inactivation varied between treatments or cells were in a different physiological state. Fig 5.4 shows the inactivation data for the cells used in the microarray study. Cells were subjected to inimical treatment at exponential phase (10^8 cells ml^{-1}), and the control and the first sample were taken just before and after (5min) treatment. 24 h after the treatment the culturable cell density was 10^4 cells ml^{-1}. At 48 h the third sample was taken, and the cells were

unable to be detected on plates (i.e. <100 CFU ml^{-1}). Finally the fourth sample was taken 72 h after the treatment.

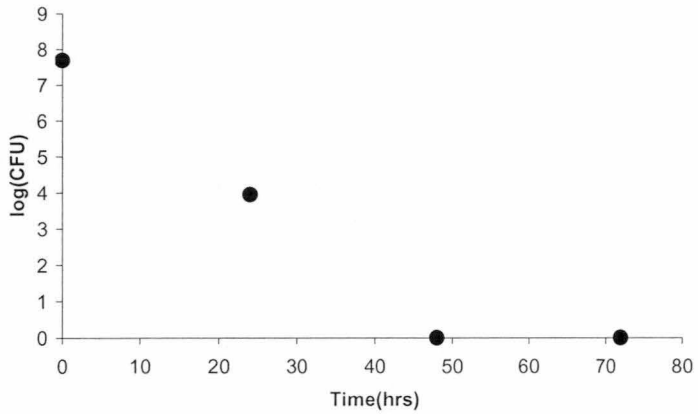


Fig 5.5 (Inactivation Trial 6) A time course study on *L. monocytogenes* ScottA inactivation in a small volume 50ml to make up 1L showing the kinetics of inactivation at pH3.5, a_w 0.90 and at 25°C.

5.4.3 Formaldehyde Agarose Gel Electrophoresis

Fig 5.5 shows an image of a gel that illustrates the RNA yield. Two large bright bands (bands 1 and 2) represent the control samples while the other lanes, which are weaker, are from inactivated samples 1 to 4 (5min, 24h, 48h, 72h-lanes 3 to 10).

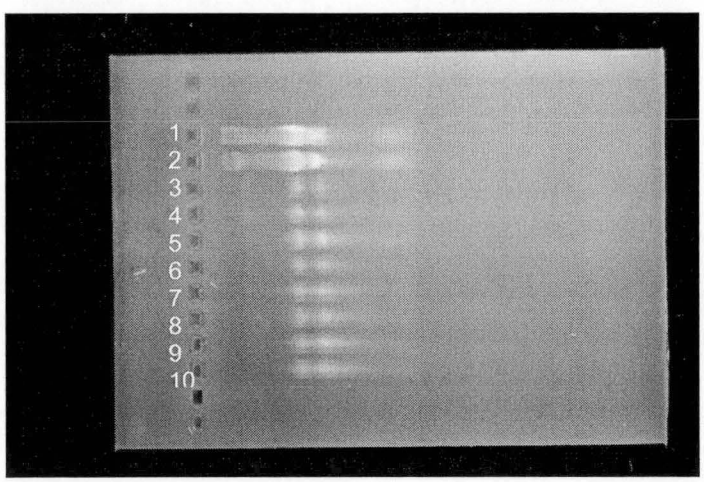


Fig 5.5 Formaldehyde Agarose Gel Electrophoresis showing results of the RNA extraction on the four different time points (control: 1 and 2; 5 min: 3 and 4; 24 h: 5 and 6; 48h: 7 and 8; 72h: 9 and 10) for Inactivation Trial 6.

5.4.4 A time course analysis: up-regulated and down-regulated genes

The salt and acid treatments applied, induced significant gene expression alterations in several gene functional categories and sub-categories, as established using the *T-value* scoring procedure of Boorsma et al., (2005). The *T-value* scoring results are summarized in Table 5.1. Gene set enrichment analysis indicated increased expression of genes associated with branched chain amino acids biosynthesis (*T-value* scores: 3.3-4.7; Table 5.1), glycine/serine/threonine metabolism (*T-value* scores: 1.8-2.7; Table 5.1), pantothenate/CoA biosynthesis (*T-value* scores: 1.3-2.3; Table 5.1), peptidases (*T-value* scores: 1.2-2.6; Table 5.1) and histidine metabolism(*T-value* scores: 0.6-2.6; Table 5.1) occurs. On the other hand, suppression of expression related to flagellar assembly (*T-value* scores: -2.1 to -3.5; Table 5.1), type III secretion (*T-value* scores: -1.7 to -2.9; Table 5.1), fructose/mannose metabolism (*T-value* scores: -0.1 to -3.0; Table 5.1) and chemotaxis (*T-value* scores: -0.77 to -1.99; Table 5.1) also occurred.

Table 5.1 *t-values* for up-regulated and down-regulated gene sets during the inactivation of *L. monocytogenes* ScottA at pH 3.5 and a_w 0.90.

Functional category	#Genes	TP1	TP2	TP3	TP4
Branched chain amino acids biosynthesis	16	**3.91**	**3.35**	**4.74**	**3.34**
Histidine metabolism	14	**2.61**	1.63	0.65	1.63
Peptidases	45	**2.58**	1.59	1.91	1.17
Pantothenate/CoA biosynthesis	15	**2.26**	1.26	**2.35**	1.76
Glycine/serine/threonine metabolism	26	**2.21**	**2.38**	1.83	**2.74**
Flagellar assembly	27	**-3.50**	**-2.83**	**-3.13**	**-2.10**
Fructose/Mannose metabolism	68	**-2.96**	-1.15	-1.02	-0.15
Type III secretion	11	**-2.57**	**-2.34**	**-2.89**	-1.65
Chemotaxis	13	-1.99	-0.77	-1.09	-1.30

Bold type for significance results $p<0.05$. TP=Timepoint

5.4.5 Time course analysis: strongly expressed genes

Genes that maintained relatively strong expression (*A values* average >13) under simultaneous low pH and low a_w code L-lactate dehydrogenase (lmo0210), single-stranded DNA-binding protein (lmo0045) and non-heme iron-binding ferritin (lmo0943), respectively (Table 5.2). Other strongly expressed genes involved in general stress responses, such as ribosomal proteins (lmo0248, lmo0249, lmo0250, lmo2741, lmo2745, lmo1335, lmo0251, lmo0211, lmo1540, lmo2655), cold shock protein (lmo2016, lmo1364), cell division protease (lmo0220), septum location regulation (lmo0197), putative cyanate permease (lmo0947), LacI family related genes (lmo2737), peptide related genes (lmo0049), PTS transporters (lmo2683, lmo2733), ribonucleotide reductase (lmo0279), RNA polymerase (lmo0259), K-uptake system (lmo1023), phosphate-binding enzyme (lmo1760), flagellin (lmo0690), EF-Tu (lmo2653) and uncharacterised transporters (lmo2741, lmo2745), which are shown in Table 5.2.

Table 5.2 Thirty strongly expressed genes during acid/salt inactivation of *L. monocytogenes* ScottA

ORF_name	Gene homolog	Regulon	Predicted or know function	A*1	A2	A3	A4
lmo0249	rplA	$hrcA^+$	ribosomal protein L1	13.91	12.03	13.19	12.55
lmo2016	cspB		similar to cold shock protein (beta-ribbon, CspA family)	13.48	12.96	12.39	12.46
lmo0943			non-heme iron-binding ferritin	13.33	12.88	13.47	13.54
lmo0045	ssb	$hrcA^+$	single-stranded DNA-binding protein	13.19	12.43	14.36	13.27
lmo0248	rplK	$hrcA^+$	ribosomal protein L11	12.86	10.90	11.61	11.57
lmo0250	rplJ		ribosomal protein L10	12.82	10.41	11.94	11.26
lmo1364	cspL	$sigB^-$	similar to cold shock protein (beta-ribbon, CspA family)	12.72	10.34	12.06	12.11
lmo0210	ldh	$sigB^+$	L-lactate dehydrogenase	12.72	13.53	14.20	13.68
lmo2741			similar to drug-efflux transporters	12.66	11.50	12.59	12.21
lmo2745			similar to ABC transporters, ATP-binding protein	12.57	11.35	12.58	12.03
lmo1335	rpmG		ribosomal protein L33	12.42	12.00	13.08	12.28
lmo0220	ftsH		cell division protease	12.39	10.69	12.79	11.73
lmo0251	rplL		ribosomal protein L7/L12	12.26	10.86	11.96	11.47
lmo0211	ctc/rplY	$sigB^+$	ribosomal protein L25 (general stress protein Ctc)	12.20	11.98	12.11	11.71
lmo0197	spoVG		similar to uncharacterized protein in the regulation of septum location	12.15	11.06	11.22	11.22
lmo0947			putative cyanate permease	11.93	11.32	10.14	10.49
lmo2707			unknown protein	11.87	10.93	13.07	11.80
lmo1934	hup	$rpoN^-$	similar to non-specific DNA-binding protein HU	11.78	9.99	10.99	10.66
lmo2683			similar to PTS system, cellobiose-specific IIB component	11.66	10.75	12.52	11.58
lmo2737			putative transcriptional regulator, LacI family	11.62	11.71	11.48	11.32
lmo2733			similar to PTS system, fructose-specific IIABC component	11.54	11.29	11.17	10.75
lmo0279			ribonucleotide reductase	11.49	11.18	11.60	10.53
lmo1540	rpmA		ribosomal protein L27	11.42	10.36	10.26	10.72
lmo2655	rpsG		ribosomal protein S7	11.35	12.10	11.42	11.29
lmo0259	rpoC		RNA polymerase, beta' subunit	11.33	9.77	10.06	9.61
lmo1023	trkA		similar to a bacterial K(+)-uptake system	11.23	10.10	9.90	9.59
lmo1687			similar to uncharacterized conserved proteins	11.13	9.87	9.12	9.19
lmo1760			predicted phosphate-binding enzyme	11.07	10.83	10.80	10.65
lmo0690	flaA	$codY^+$	flagellin	11.02	11.27	11.63	12.21
lmo0049	agrD	$rpoN^-$, $codY^-$	putative auto-inducing peptide	11.01	9.04	11.21	9.87
lmo2653	tufA		elongation factor EF-Tu	10.08	10.76	9.85	9.70

* Total spot intensity A=($\log_2$ Cy3+$\log_2$ Cy5)/2 following normalization.

5.5 DISCUSSION

L. monocytogenes ScottA has evolved sophisticated mechanisms to cope with environmental stresses (Raengpradub et al., 2008; Bennett et al., 2007; Arous et al., 2004; Milohanic et al., 2003). This study focused on a molecular mechanisms related to combined of acid (pH3.5) and salt (a_w 0.9) induced inactivation of exponential phase cells.

5.5.1 The possible explanation for the difference between large (1L) and small (50ml) volume inactivation

It was observed that the inactivation kinetics of a 1L volume culture (Fig 5.3 a-d) were found to be totally different from those observed in 50ml cultures (Fig. 5.4 and 5.5), the former showing immediate inactivation after the acid and salt treatment, compared to the 50 ml cultures in which populations lost culturability slowly over 48 h. One possible reason for the apparently rapid inactivation of large volume (1L) might be that because clumps were formed at the bottom of the flask, each and every cell in the population was potentially not homogenously inactivated; Fig 5.3b shows the same phenomenon. Direct mixing of concentrated acid and salt to the exponential phase culture appears to cause rapid inactivation possibly owing to the sensitivity of the cells, unlike stationary growth phase cells, which are notably more acid tolerant (Davis et al., 1996). This conclusion was reinforced in the experiment where centrifugation and resuspension of the log phase cells in 1ml broth at pH 3.5 and a_w 0.9, obtaining a very high cell density, before inoculation to the larger volume rendered the same result (Fig. 5.3d). Utilising small volume (50ml) inactivation, however, the kinetics of the inactivation was different, with cells

becoming undetectable by culture only after 48h (Fig. 5.4).

A possible explanation for the different results observed might be due to slight differences in the physiological state of cells in the different experiments. The proportion of tolerant cells may be greater in inactivation experiment 5 possibly because a higher number of cells have entered into or were already in an acid tolerant state (Davis et al., 1996; Datta and Benjamin, 1997; Samelis et al., 2003) at the time of the treatment e.g. Fig. 5.4, i.e. due to having entered stationary phase. Further investigation is still needed to elucidate this phenomenon.

5.5.2 Changes in gene expression in functional gene sets

In the experiment described in Section 5.3.2 (Inactivation Trial 6), branched chain amino acids (BCAAs) are the most strongly up-regulated gene set with *T value* >3 and significance $p<0.05$ for all four time points results (Table 5.1). In addition to this histidine metabolism, glycine/threonine/serine metabolism and panthothenate/CoA metabolism are all upregulated. Potentially these metabolic functions may be interrelated and required for the survival of cells inactivated under the experimental conditions imposed.

L. monocytogenes encodes a functional member of the CodY family of global regulatory proteins that is responsive to BCAAs. The CodY regulon encodes proteins involved in nutrient transport, genetic competence, motility and chemotaxis (lmo0690 is positively regulated by codY, flaA encoded flagellin, Table 5.2). By sensing the levels of BCAAs, CodY gives a more specific measure of the nutritional

state of the cell, thus allowing the cell to respond subtly to different nutritional and environmental stresses, i.e. CodY plays an important role in *L. monocytogenes* in regulating *de novo* biosynthesis of amino acids in response to the nutritional state of the cell (Sonenshein, 2005; Bennett et al., 2007). Thus, CodY is a pleiotropic repressor sensing nutritional supply as a function of the BCAA pool in the cell; therefore, BCAAs play a central role as a signal for CodY activity (Guedon et al., 2001).

Histidine metabolism associated genes

In response to the physiological state of the cell, CodY regulon in *L. monocytogenes* comprises genes involved in amino acid metabolism, nitrogen assimilation as well as genes involved in sugar uptake and incorporation, indicating a role for CodY in *L. monocytogenes* in both carbon and nitrogen assimilation (Bennett et al., 2007), which might explain why histidine was also up-regulated in this study (Table 5.1).

Histidine has a positively charged imidazole functional group and relatively small shifts in cellular pH will change its charge (Cornishbowden, 1984b). Liu et al., (2002) reported that bacterial two-component signal transduction systems, consisting of histidine kinase sensors and DNA-binding response regulators, allow bacteria to respond to diverse environmental stimuli, such as the cold adaptation response. O'Donovan and Ingraham (1965) showed that the first enzyme in histidine biosynthesis was more sensitive to feedback inhibition by histidine at low temperatures. It is possible that *L. monocytogenes* cells become starved for certain amino acids at least partially through feedback inhibition and respond by inducing

biosynthetic enzymes for histidine and aromatic amino acids. In a histidine proton shuttle, histidine is used to quickly shuttle protons by abstracting a proton with its basic nitrogen to make a positively-charged intermediate and then use another molecule, a buffer, to extract the proton from its acidic nitrogen (Cornishbowden, 1984a).

Serine/threonine/glycine metabolism

In catalytic triads, the basic nitrogen of histidine is used to abstract a proton from serine/threonine to activate it as a nucleophile. In current studies, the serine/glycine biosynthetic pathway was up-regulated, with three out of four time points showing significant up-regulation ($p<0.05$) (Table 5.1), where serine is the precursor to the simplest amino acid-glycine. Degradation of exogenously supplied serine to acetate has previously been shown to occur in association with the heat shock response (Matthews and Neidhardt, 1989). Gschaedler and Boudrant (1994) reported that glycine and threonine were the only "energy amino acids", which are catabolised to provide energy, that increased the specific growth rate of *E. coli*. Whether *L. monocytogenes* can energize by a similar process remains to be determined.

Pantothenate synthesis

Glycine can be directly incorporated into cellular protein, converted to threonine and to serine (Marcus and Dekker, 1993), and fed into the glycolytic pathway via degradation of serine to pyruvate and acetyl CoA, an acyl group carrier in the tricarboxylic acid cycle. Pantothenate (vitamin B_5) synthesis was found to be up-regulated in treated cells (Table 5.1). Pantothenate, in a five-step process, leads to

the production of coenzyme A (CoA), notable for its role in the synthesis and iodation of fatty acids, and the oxidation of pyruvate in the citric acid cycle (Rock et al., 2000). Genes coding for pantothenate kinase (PanK) are the control step to the pathway of pantothenate/CoA biosynthesis (Begley et al., 2001). The feedback inhibition of PanK activity by CoA and its derivatives represents a key regulatory mechanism that controls intracellular CoA levels in response to the metabolic status of a cell (Leonardi et al., 2005; Yang et al., 2006).

Peptidases

To survive, nucleotide biosynthesis, initiation of protein translation and protein synthesis events, *L. monocytogenes* cells require one-carbon units and amino acids (Cornishbowden, 1984b). Peptidases, or proteases found to be, up-regulated (see Table 5.1), catabolize proteins by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain. They may also break specific peptide bonds, depending on the amino acid sequence of a protein, or break down a complete peptide to amino acids (van Wely et al., 2001).

Several bacterial ribosomal proteins were strongly expressed in this study (Table 5.2) and suggest that they are necessary to resume normal peptide synthesis. These ribosomal proteins have also been found to be associated with cold shock stress (Chan et al., 2007). Signal peptidases (SPases) (Paetzel et al., 2000) are required for the targeting of precursor proteins to the cytoplasmic membrane, and for the initiation of their translocation across this membrane (Pugsley and Possot, 1993). In Gram-positive bacteria, the exported proteins are either secreted into the medium,

or they remain associated with the bacterial envelope (Cabanes et al., 2002; Glaser et al., 2001). Thus, by complex cooperative action the peptidases may proceed as cascade reactions, which result in rapid and efficient amplification of an organism's response to a physiological signal.

When growth slows down and inactivation starts, whether this depends on lethal acid or salt stress (Sitnikov et al., 1996; Withers and Nordstrom, 1998), the requirement of amino acids and one-carbon units is increased to enable cells to survive and to maintain physical integrity. An increased degradation of BCAA under stress conditions could provide an alternative carbon source or detoxification mechanisms by maintaining the pool of free branched-chain amino acids at levels compatible with cellular homeostasis (Harris et al., 2004).

5.5.3 Microarray data analysis: Mobility

The most obviously down-regulated genes group are associated with mobility, including flagellar assembly ($p<0.05$ at four time points), type III secretion ($p<0.05$ at three time points), and chemotaxis (Table 5.1), all of which are systems that enable cells to direct their movement to certain chemicals in their environment. Flagella comprise long and rigid filaments that acts as a propeller anchored to the cell envelope by a flexible hook and basal body (Bigot et al., 2005).

The structure of Type III secretion systems (TTS) shows that a needle complex spans inner and outer membranes and comprises a cylindric base with a thin, needle-like structure projecting outwards from the cell, in many ways resembling

the basal body of the flagellar apparatus (Kubori et al., 1998; Blocker et al., 1999). Thus, TTS systems may have evolved from flagellar assembly systems. Bigot et al. (2005) reported that FliF, the flagella basal body component, and FliI, a protein similar to the ATPase, are essential for flagella assembly in *L. monocytogenes*. Transcription of flagellar genes generally occurs in a hierarchical fashion that mirrors assembly of the nascent flagellum, the genes encoding the early flagellar components being transcribed prior to the genes encoding late flagellar components such as flagellin subunits (Kutsukake et al., 1994).

It has been suggested that PrfA would participate in the down-regulation of motility genes in *L. monocytogenes* at 37°C (Michel et al., 1998). A more recent study has shown that another regulatory protein, MogR, directly binds to the FlaA promoter region (Grundling et al., 2004). MogR acts as a repressor of motility gene expression during intracellular infection and is required for full virulence of *L. monocytogenes*. In this study, *flaA,* lmo0690, that encodes the *L. monocytogenes* major flagellin protein FlaA and is positively regulated by codY, maintaining strong expression as shown Table 5.2.

Raengpradub et al., (2008) reported that σ^B negatively regulates genes encoding proteins related to motility and chemotaxis in response to salt stress. Lmo1699 (encoding a protein similar to methyl-accepting chemotaxis proteins) which represents an operon within the DegU regulon (the two-component response regulator), were found to be negatively regulated by σ^B in salt-stressed cells. Eighteen flagellum-specific genes and three chemotaxis-specific genes were also found to be negatively regulated by σ^B in salt-stressed cells and there was no

evidence that HrcA regulates flagellar genes (Hu et al., 2007a). It was also observed that genes involved in flagellar biosynthesis were derepressed in a codY mutant (Bennett et al., 2007).

5.5.4 Strongly expressed genes

Bacterial survival under stress conditions requires rapid alterations in gene expression, controlled by direct and indirect transcription of the association of different regulons in response to cellular signals. Four of the regulons involved in this study are SigB, CodY, RpoN and HrcA (Table 5.2).

L-lactate dehydrogenase (lmo0210)

σ^B, encoded by *SigB*, has been recognized as a general stress responsive sigma factor that contributes to survive under several adverse conditions, such as high osmolarity, low temperature, and acidity (Hu et al., 2007b). In this study, σ^B was found to positively regulate *ldh* which encodes L-lactate dehydrogenase (L-LDHs) (lmo0210) and was one of the most strongly expressed genes (Table 5.2). It is an enzyme that catalyzes the interconversion of lactate and pyruvate. Bacterial L-LDHs exhibit a wide variety of high amino acid sequence divergence (Griffin et al., 1992). Lactate metabolism is believed to protect the organisms from sugar killing, perhaps through more rapid movement of carbohydrate through glycolysis, more efficient movement of lactic acid out of the cell compared with other organic acids, and maintenance of NAD/NADH balances. In reducing pyruvate to L-lactate, NADH is oxidized to NAD and the redox potential of the cells is preserved (Arai et al., 2002) and the conversion of pyruvate into acetoin helps to maintain pH

homeostasis (Monnet et al., 2003).

Nonheme iron-binding ferritin (lmo0943) and *ctc* (lmo0211)

Raengpradub et al., (2008) reported that *fri* (lmo0943) which encodes Fri, a nonheme iron-binding ferritin (lmo0943), and the general stress response gene *ctc* (lmo0211), are positively regulated by σ^B under salt-stressed *L. monocytogenes*. These genes were also observed to be relatively highly expressed in this study for combined salt and acid stresses (Table 5.2). It is noteworthy that growth of *Campylobacter jejuni* was significantly reduced during cold acclimation when the induction of ferritin was prevented (Wai et al., 1996). Chan et al. (2007) also reported that σ^B activity is induced during 30min of cold shock, and transcription of the putative cold stress genes *fri* is σ^B independent during growth at 4°C. Thus, the ferritin like protein (Flp) may act as a multi-stress protein, the induction of which responds to very different stressing agents.

Single-stranded DNA-binding protein (lmo0045)

σ^B appears to contribute to direct regulation of hrcA transcription, indicating that HrcA is part of an integrated network of transcriptional regulators contributing to stress response systems in *L. monocytogenes* (Hu et al., 2007a). In this study, HrcA regulated genes that were highly expressed encode two ribosomal proteins (L11 and L1, lmo0248-lmo0249) and SSB (lmo0045, Table 5.2), which binds single-stranded DNA with high specificity.

The SSB protein has been found to be involved in the main pathways of

reproduction and maintenance of genetic information (Molineux and Gefter, 1975). *In vivo*, Meyer et al. (1979) reported that the SSB protein is essential for replication and it also participates in recombination and repair processes (Glassberg et al., 1979). In several cases, it has a direct influence on enzymatic activities of DNA specific proteins (Kunkel et al., 1979). Also, it stimulates the strand annealing activity of the recA protein (McEntee et al., 1980). *In vitro*, SSB enhances several molecular biology applications by destabilizing DNA secondary structure and increasing the processivity of polymerases (Glassberg et al., 1979; Meyer et al., 1979). Raengpradub et al. (2008) reported that SSB was not associated with acid tolerance. Thus, from the results of this study, it is hypothesized that the strong expression of SSB could be associated with salt tolerance.

Cold shock protein (lmo1364)

Hebraud and Guzzo (2000) have shown that members of cold shock proteins (Csps) are small RNA-binding proteins that mediate transcription elongation and message stability. In this study, σ^B was also suggested to contribute to negative regulation of gene transcription, likely through indirect means, i.e. through positive regulation of a repressor, such as *cspL*, which encodes similar to cold shock protein (beta-ribbon, CspA family, Table 5.2). Liu et al. (2002) reported that members of the CspA family, have received considerable attention in studies of the bacterial cold shock responses and they are required for cold acclimation.

Phosphotransferase system (lmo2683 and lmo2733)

In this study, lmo2683 and lmo2733, which encode PTS system enzymes, including

cellobiose-specific IIB component and fructose-specific IIABC component, are strongly expressed (Table 5.2). Raengpradub et al., (2008) also reported that operons of *L. monocytogenes* positively regulated by σ^B are involved in carbohydrate metabolism include a mannose-specific PTS operon, a fructose-specific PTS and an operon that includes genes encoding enzymes in the pentose phosphate pathway. PTS activity is known to have a direct effect on the pyruvate pool and on catabolite regulation (Williams et al., 1986).

Bacterial K^+ uptake system (lmo1023) and Compatible solutes

Compatible solutes are soluble organic compounds that can reach high intracellular concentrations without disturbing vital cellular functions. To maintain cell turgor, the pooling of compatible solutes is a strategy for dealing with hyperosmotic conditions in bacteria (Kempf and Bremer, 1998). Also, increase in the intracellular levels of K^+ is the first response of many microorganisms to an increase in extracellular osmolality. McLaggan et al. (1994) reported that genes sharing homology with *trkA* (trkA, lmo1023, encodes a protein similar to that involved in the bacterial K^+ uptake system and was strongly expressed, Table 5.2) are involved in the transport of K^+ into cell. σ^B facilitates the transport of compatible solutes during salt osmotic stress by regulating various transport proteins coding genes i.e. betL, sbu operon, opu operon (Moorhead and Dykes, 2003).

Elongation factor EF-Tu (lmo2653)

In Table 5.2, lmo2653 sharing the *tufA* homolog, encoding elongation factor EF-Tu is strongly expressed and it is an expected result since it is an important

housekeeping gene, and is consistent with the results and conclusions of Chapter 4 that total *tuf* gene expression stays relatively stable despite that cells were inactivated at pH3.5/a_w0.9. The other genes displayed in Table 5.2 are also consistently expressed over the same time points. This observation suggested that cells are metabolically active despite being unable to be cultured on agar plates. Thus, together with the findings in Chapter 4, culturability and viability appear to be distinct concepts in the regards to non-thermal inactivation. This chapter sought to identify what genetic responses are associated with a shift from an environment that supports growth to one that is inimical to growth and basically provides a view of *L. monocytogenes* when it is rendered into an apparently viable but not culturable state.

5.6 CONCLUSION

The aim of this study was to further investigate the mechanism of high salt (a_w 0.9) and acid (pH 3.5) concentration on the inactivation of *L. monocytogenes* ScottA in a time course study (5min, 24h, 48h, 72h) and using genomic approaches. The results show that a large number of genes for amino acid biosynthesis and metabolism are up-regulated, possibly indicating a switch to alternative carbon sources as energy sources and to ensure cell integrity. Genes belonging to the categories of structure and function of cell wall, cell movements and carbohydrate metabolism were down-regulated indicating lowered mobility. Genes that were maintained at comparatively strong expression levels suggest that they play important roles in the survival of *L. monocytogenes* under high salt and acid stress levels. Proteomic studies for detection unknown regulons and/or proteins and the utilisation of the CTC/DAPI microscopic double staining technique would be useful approaches to deduce the presence of viable but not culturable cells and to further explore the mechanisms of non-thermal inactivation in *L. monocytogenes*.

GENERAL SUMMARY AND CONCLUSION

To test the hypothesis that non-thermal temperature is the main factor that governs the inactivation of vegetative bacteria when other factors prevent their growth, this project initially investigated the kinetics of inactivation of two bacterial species, *E. coli* and *L. monocytogenes*, when two environmental parameters: pH and a_w prevented growth. Temperature, pH and water activity are the most influential factors affecting the survival of microorganisms, and the most common hurdles used in food preservation.

The results suggest that the inactivation rate responses and kinetics of both microorganisms are not systematically different which suggests that the influence of non-lethal temperature on the rate of inactivation of vegetative bacteria in inimical environments is not species-dependent. If correct, this observation could greatly simplify the ability to assess the safety of some food processes, e.g. fermentation, in which no overtly lethal treatment is applied. To further assess the hypothesis, investigation of other inimical, non-thermal, stresses, other bacterial species and in other food products, such as cheese, is needed. Ross *et al.* (2008) suggested that the effect of inimical conditions is not strongly dependent on the severity of the conditions applied, with most variation in inactivation rate being explained by temperature variations. Accordingly, the support for that hypothesis provided by the current study raises the question of the mechanism of that process of inactivation. This was also explored, as discussed further below.

Following the kinetics study, two rapid alternative indirect enumeration methods, cellular ATP content *via* bioluminescence measurement and QPCR, were compared with viable count enumeration methods. While viable counts methods (i.e. "plate counts") continue to be widely used for microbial enumeration they are slow and labour– and space-intensive.

Intracellular ATP measurement is an alternative enumeration method that is both rapid and sensitive, and its utility as an indicator of microbial inactivation was investigated to facilitate aspects of this PhD study. Luminometry and viable count were studied in parallel. However, the result shows that ATP method is not significantly well correlated with viable counts of non-growing cells to quantity microbial inactivation but is well correlated with microbial growth. Thus, luminometry may only be applied to actively growing cells as a labour and material saving method for quick estimation of the size of viable populations. However, a more interesting observation was that, even when no colonies grew on the plates, there was still a certain amount of bioluminescence, suggesting that cells may still be alive but too "damaged" to reproduce. To explore another alternative enumeration method, but more particularly to explore the physiological state of cells in inimical environments, *tuf* gene expression in *L.monocytogenes* was assessed. The *tuf* gene encodes a protein elongation factor and is constitutively expressed.

L. monocytogenes exposed to pH3.5/a_w0.9 at 25°C, resulted in a more than 10^8 decline in viable cells, but total *tuf* gene expression assessed by the mRNA signal, remained

stable. Thus, as with ATP bioluminescence analysis, mRNA levels also suggested relatively high levels of ongoing metabolic activity in the absence of culturable cells. One interpretation of these results is that intracellular ATP and that the level of expression of the housekeeping gene *tufA*, are not constant properties within the cell. Another possibility, however, is that inimical conditions cause cells to become unculturable and, thus, uncountable by traditional methods despite maintaining their viability, i.e., that inimical conditions induce the so-called 'viable but non-culturable state '(VBNC) in *L. monocytogenes*. To further investigate the question whether cells in non-growth conditions are still alive and actively maintaining their structure and function, or are metabolically inert and dead or dying, studies involving antibiotic and high temperature challenge were undertaken.

L. monocytogenes populations were inactivated with 55°C and/or rifampin and *tuf* gene expression subsequently assessed by QPCR. *tuf* gene expression was reduced one thousand-fold in the presence of rifampin compared with only a half log reduction with the mildly lethal temperature. Therefore, *L. monocytogenes* may still be metabolically active after being rendered non-culturable under mildly lethal conditions, such as pH, a_w or mildly super-maximal temperature.

The two *L.monocytogenes* strains, employed in Chapter 3 and 4, are both of serotype 4B. *L. monocytogenes* Scott A is a type strain and *L. monocytogenes* Fw03/0035 is a salt resistant strain. The purpose of the ATP method is to find an alternative method to enumerate microbial inactivation compared with viable count. The results in Fig 3.3

and Fig 3.4 show that there is little variation between these two strains, thus strains difference account for little influence on the observed responses. In chapter 4, the QPCR method is also investigated as a rapid method for enumeration of microbial inactivation, and the other purpose was to explore the mechanisms of inactivation to answer question whether cells in non-growth environment are still alive and maintaining their structure or function and are they metabolically inert. Fig 4.2 showed variation of inactivation kinetics between these two strains by viable count, but there was little variation observed in mRNA signals which remain stable during the *L. monocytogenes* inactivation process. On the basis of those, and other, results, were not consistent enough to be an important source of variability.

To address the question of mechanisms of non-thermal inactivation, further studies of cell physiology were undertaken, as described in Chapter 5, using DNA microarray analysis techniques. Gene expression over time, during inactivation of *L. monocytogenes* at pH 3.5 and a_w 0.9, was assessed from 5 minutes after imposition of the inimical conditions until 72 hours later.

The results suggested that the majority of up-regulated genes are related to amino acid biosynthesis and metabolism indicating that, to ensure cell integrity under lethal stress conditions, cells might switch to alternative carbon sources as energy source. Genes were consistently expressed over the time points. Thus, cells from normal living conditions switch to a so-called "hibernation" condition when under growth-preventing osmotic or acid stress, presumably adopting alternative energy sources while trying to

maintain their integrity and also reducing energy spent on motility. From the number of genes maintaining constantly high expression during the exposure to inimical conditions, house keeping genes are suggested to play an important role on the maintenance of cellular activity.

The principle conclusion that can be drawn from the whole thesis is that when vegetative bacteria cells encounter non-thermal lethal conditions, such as low pH and water activity, or combinations thereof, that house keeping genes start to up-regulate or down-regulate to repair the damage occurring, reduce energy cost and maintain residual cellular activity. During the process of inactivation, inactivation rates were determined mainly by temperature conditions rather than other environmental factors and when they reach the point of completely losing their culturability, they may still remain metabolically active, thus enter the state of viable, but non-culturable, cells.

The results add evidence to the thesis that culturability and viability may be distinct concepts in regard to non-thermal inactivation. To further assess the presence of viable but not culturable cells, proteomic studies and the CTC/DAPI microscopic technique would be useful to enhance knowledge of the mechanisms of non-thermal inactivation.

For practical purpose, the results add great value to understand safety, processing and preservation of non-thermal processed foods. When boiling or chilling is not the preferred way to make the food safe, such as salami and cheese, other hurdles are relied upon to make the foods. During the process of the bacteria inactivation, because of the

other hurdles, the main factor influencing food safety during the processing and storage is the temperature. Nowadays, researchers and food companies focus a lot on the combination of hurdles to make the food safe, but this thesis shows when the other factors can kill bacteria, or make it inactivated, the main factor that affects the rate of bacteria inactivation is temperature. This knowledge can be used to improve the design of formulations and processes to enhance the safety of such foods.

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APPENDIX A

A.1 STRAIN DETAILS, MAINTENANCE AND RECOVERY

A.1.1 Bacterial strains

E. coli M23

shown to be non-pathogenic due to the absence of virulent markers, and from cryogenic culture, courtesy of Dr K Sanderson, University of Tasmania, Private Bag 54, Hobart, TAS, 7001

E. coli R31

Verotoxigenic clinical non-typable strain from slope culture courtesy of Dr. S. Bettiol, Clinical School, University of Tasmania

E. coli SB1

shown to be non-pathogenic due to the absence of virulent markers, and from clinical isolate, courtesy of Ms S. Bettiol, Department of Pathology, University of Tasmania.

E. coli MG1655

K12, non-pathogenic Datsenko and Wanner (2000), from the School of Agriculture Science culture collection, University of Tasmania, GPO Box 252/54, Hobart, TAS, 7001

L. monocytogenes ATCC19115

isolated from a human clincal sample (cerebrospinal fluid) from the American Type Culture Collection, Manassas, Virginia, USA. Serotype is 4b.

L. monocytogene ScottA

isolated from a human outbreak in an epidemic in Massachussetts (USA) in 1983 also serotype 4b = CIP 103575 Purchased from ATCC. (CIP = Pasteur Institute collection of microorganisms).

L. monocytogene Fw03/0035

isolated from a processed meat product, provided by Dr. Agnes Tan, Medical Diagnostic Unit, University of Melbourne, Parkville, VIC. Serotype is 4b.

A.1.2 Cryogenic storage

Bacterial cultures for all experiments were stored at -70°C. Cultures were maintained in duplicate, with one set held for subculture purposes only.
Each *E. coli* strain was grown for 14 hours on Tryptone Soya Agar (TSA) at 37°C. The plate was harvested by pipetting 1ml of sterile Nutrient Broth with 15% glycerol added (NB-Gly) onto the surface of the plate then emulsifying the growth with a wire loop.

While each *L. monocytogene* strain was grown for 24 hours on Brain Heart Infusion Agar (BHA) at 37°C The plate was harvested by pipetting 1ml of sterile Brain Heart Infusion broth with 30% glycerol added (BHI-Gly) onto the surface of the plate then emulsifying the growth with a wire loop

The bacterial suspension was aseptically pipetted into two vials. Vials were placed at -20°C for 24 hours before transfer to -70°C for storage up to 7 years.

A.1.3 Recovery from cryogenic storage

E. coli cultures were recovered by aseptically removing the cells from the surface of

the thawed stock culture using a sterile yellow pipette tip, plated to TSA and incubated for 14 hours at 37°C. The culture was checked visually for purity (colony morphology only) then plated onto an appropriate medium to test for typical reactions.

L. monocytogenes cultures were recovered by aseptically removing the cells from the surface of the thawed stock culture using a sterile yellow pipette tip, plated to BHA and incubated for 24 hours at 37°C. The culture was checked visually for purity (colony morphology only) then plated onto an appropriate medium to test for typical reactions.

A.2 MEDIA

A.2.1 Media preparation

Basal media was prepared as per manufacturers instructions unless otherwise stated. Addition of supplements was as per manufacturers instructions or as described in this section. Sterilisation was by autoclaving at 121°C at 106K Pa for 15 minutes (unless otherwise specified) or in the case of non-sterile, heat sensitive supplements by filter sterilization. Where necessary pH was modified by the addition of 0.1M NaOH or 0.1M HCl unless otherwise stated. pH was measured post-autoclaving and adjusted aseptically by the addition of sterile HCl or NaOH if required. For NaCl modified media, a_w was determined from triplicate readings. All water used in the preparation of media was prepared by glass distillation of tap water. Media were stored at 4°C for up to 4 weeks. Media containing antibiotics was stored in the dark at 4°C for up to 2 weeks.

A.2.2 Culture Media

Brain Heart Infusion Agar (BHA)

Brain Heart Infusion Broth (Oxoid CM225)	37.0g
Technical Agar No.3 (Oxoid L13)	15.0g
Distilled Water	1000ml

After autoclaving, agar medium was cooled to 50°C prior to pouring plates.

Brain Heart Infusion Agar with 0.1% Pyruvate (BHA-P)

Brain Heart Infusion Broth (Oxoid CM225)	37.0g
Technical Agar No.3 (Oxoid L13)	15.0g
Pyruvic Acid (Sigma P-8574)	1.0g
Distilled Water	1000ml

After autoclaving, agar medium was cooled to 50°C prior to pouring plates.

Brain Heart Infusion Broth (BHI)

Brain Heart Infusion Broth (Oxoid CM225)	37.0g
Distilled Water	1000ml

Brain Heart Infusion Broth at various a_w (BHI/ a_w)

Brain Heart Infusion Broth (Oxoid CM225)	37.0g
NaCl	% w/w
Distilled Water	1000ml

Over-strength BHI was prepared in less than 1000ml of distilled water in a volumetric flask, to which the appropriate amount of NaCl (determined by reference to Table 3 in Chirife and Resnik, 1984) had been added by weight. The medium was autoclaved, and then sterile distilled water was added to make the final volume up

to 1000ml.

Tryptone Soya Agar (TSA)

Tryptone soya broth(Oxoid CM 129)	30g
Technical Agar No.3 (Oxoid L13)	15.0g
Distilled H_2O	1000ml

After autoclaving, agar medium was cooled to 50°C prior to pouring plates.

Tryptone Soya Broth (TSB)

Tryptone Soya Broth (Oxoid CM 129)	30.0g
Distilled Water	1000ml

Tryptone Soya Broth at variable a_w (TSB/ a_w)

Tryptone Soya Broth (Oxoid CM 129)	30.0g
NaCl	% w/w
Distilled Water	1000ml

Over-strength TSB was prepared in less than 1000ml of distilled water in a volumetric flask, to which the appropriate amount of NaCl (determined by reference to Table 3 in Chirife and Resnik, 1984) had been added. The medium was autoclaved, then sterile distilled water was added to make the final volume up to 1000ml.

Tryptone Soya Broth + 0.6% Yeast Extract (TSB-YE)

Tryptone Soya Broth (Oxoid CM 129)	30.0g
Yeast Extract (Oxoid L21)	6.0g
Distilled Water	1000ml

Tryptone Soya Broth + 0.6% Yeast Extract at variable a_w (TSB-YE/ a_w)

Tryptone Soya Broth (Oxoid CM 129)	30.0g
Yeast Extract (Oxoid L21)	6.0g
NaCl	% w/w
Distilled Water	1000ml

Over-strength TSB-YE was prepared in less than 1000ml of distilled water in a volumetric flask, to which the appropriate amount of NaCl (determined by reference to Table 3 in Chirife and Resnik, 1984) had been added. The medium was autoclaved, and then sterile distilled water was added to make the final volume up to 1000ml.

A.3 CHEMICAL REAGENTS AND SOLUTIONS

A.3.1 Chemicals and Kits

- Pyruvate acid P8574 Sigma Australia
- Xylenol Blue 205486 Sigma Australia
- ENLITEN® ATP Assay System FF2000 Promega USA
- QuantiTect® SYBR® Green RT-PCR Kit Qiagen USA
- Quant-iT™ RiboGreen® RNA Assay Kit R11490 Molecular Probes USA
- RNAeasy® Protect Bacteria Mini Kit Qiagen USA

The remaining chemicals employed throughout this study were obtained from a variety of commercial distributors.

A.3.2 Reagents and Solutions

ATP Assay buffer

Tris	20mM
EDTA	2mM

(pH 7.75, using 2M KOH).

5%TCA/EDTA solution

trichloroacetic acid	5%
EDTA	4mM
xylenol blue dye	0.0005% to 0.002%

10%TCA/EDTA solution

trichloroacetic acid	10%
EDTA	4mM
xylenol blue dye	0.0005% to 0.002%

A.3.3 SOFTWARE

- CIA-BEN Version 2.2 from Spiral Biotech (USA) was used for bacterial colony image analysis.
- Microsoft® Excel 2000 from Microsoft Corporation (USA) was used for data presentation and statistical analysis.
- Kodak 1D Limited Edition 3.5 from Kodak (Australia) was employed to acquire CDNA gel images.
- GLM Version 2.8 from Gemini Data Loggers (UK) was used in the monitoring of temperature.
- Rotor Gene™ 6000 operating software from Corbett Research Pty Ltd (Australia) was used for Real Time PCR image analysis.

A.4 EQUIPMENT

A.4.1 Autoclave

Pressure cooker RY-150 from Rinnai (Australia) used with countdown timing systems.

A.4.2 Centrifuges

- 'Easyspin' bench-top centrifuge, Sorvall® Instruments DuPont, Dupont Company, Biotechnology Systems Division, Wilmington, DE 19898, USA.
- Beckman J2-21 M/E Centrifuge, Beckman Instruments Inc., Spinco Division, 1050 Page Mill Road, Palo Alto, CA 94304, USA.
- Universal 16A, Imbros, Australia.
- Microcentrifuge 5417R, Eppendorf, Germany.
- Sorvall® SUPER T21, Kendro, USA.

A.4.3 Fluorometer

*Pico*fluor™ Handheld Fluorometer Version:1.2 P/N 998-0853,Sunnyvale, 94085, CA

A.4.4 Luminometer

Autolumat LB 953, EG & G Berthold, Wildbad 75323, USA.

A.4.5 pH Meter

Orion pH meter 250A (Orion Research Inc. electrode Boston, MA 02129, USA), and flat tip probe.

The instrument was calibrated on each occasion before use by reference to buffers at pH 4 and pH7.

A.4.6 Pipettes

Fixed and variable pipettors were used:

- 100µl and 1000µl-variable (Gilson Medical Electronics, B. P. 45, F95469

Villiers-le-Bel, France).

- 0.01-10.00ml-electronic digital pipette (Rainin Instrument Co., Inc., 5400 Hollis St, Emeryville, CA 94608-2508).

The dispensed volume of all pipettors was checked by weighing of water at room temperature prior to use. This volume was typically found to be within ±1% of the nominal volume.

A.4.7 Rotor Gene™ 6000

Rotor Gene™ 6000, Corbett Research Pty Ltd, 14 Hilly Street, Mortlake, NSW 2137, Australia

A.4.8 Spectrophotometer

Spectronic 20, Spectronic Instruments Inc., 820 Linden Avenue, Rochester, NY 14625, USA

A.4.9 Spiral Plater

Spiral Biotech Inc., 7830 Old Georgetown Autoplate 4000 Rd, Bethesda, MD 20814 USA.

A.4.10 Stomacher

Colworth 400, A.J. Seward, London, UK. Bio- Stomacher Bags-Service Pty. Ltd., P.O.Box 180, Huntingdale, 17.7×30.0cmVic, 3166, Aus.

A.4.11 Thermometer

Fluke® 51K/J (John Fluke Mfg. Co. Inc., 1150 W. Euclid Avenue, Palatine, IL 60067, USA) electronic thermometer with Iron-Constantan thermocouple bead

probe. Calibration was checked periodically at 0°C and 100°C.

A.4.12Water Activity Meter

Aqualab CX-2 (Decagon Devices, Inc., PO Box 835, Pullman, Washington 99163, USA).

The instrument was calibrated on each occasion before use by reference to distilled water and to a saturated salt (NaCl, a_w 0.953) standard.

A.4.13Waterbaths

Patek SWB20D shaking waterbaths, Ratek Instruments Pty. Ltd., Unit 1/3 Wadhurst Drive, Boronia, VIC, Australia, 3155.

A.5 Company

A.5.1 Australian Genome Research Facility Ltd

Project ID: MACA 1090/MACA 1091
File type: .gpr and .txt
Date: 07/09/07

AGRF contact details for this project:
Name: Stephen Wilcox
Phone: 03 9345 2672
Email: Stephen. Wilcox@agrf.org.au
www.agrf.org.au

APPENDIX B

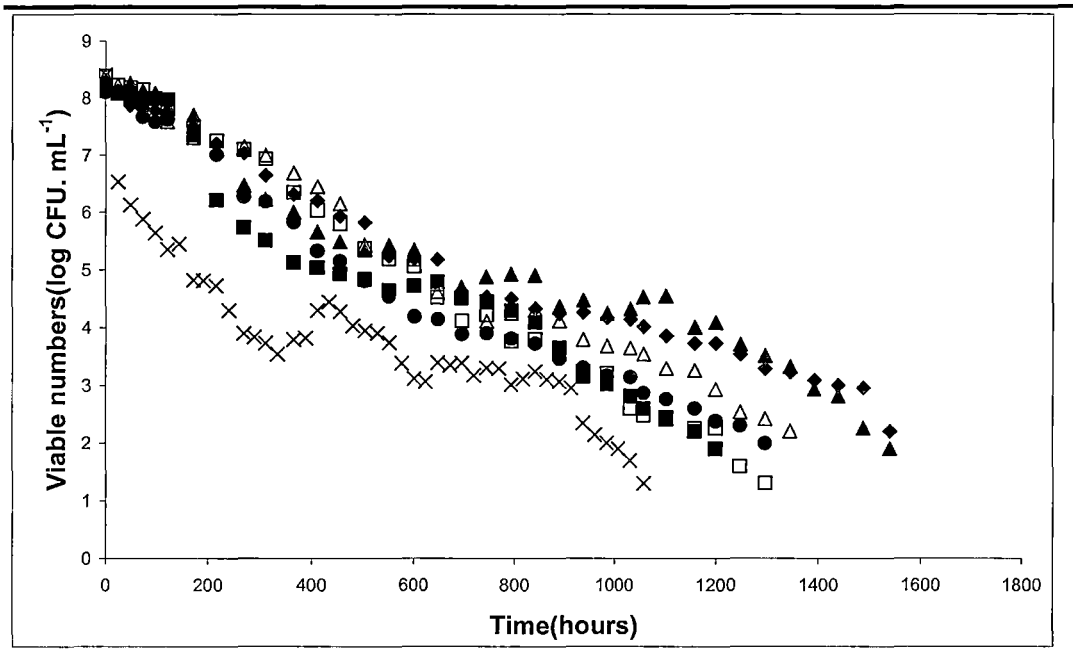

Fig B 1 (5°C) Inactivation of *E. coli* R31 (■), *E. coli* SB1 (▲), *E. coli* M23 (◆), *E. coli* MG1655 (●) and *L. monocytogenes* ATCC19115 (□), *L. monocytogenes* ScottA (x), *L. monocytogenes* Fw03/0035(Δ) in the broth model at pH 3.50 and a_w 0.900.

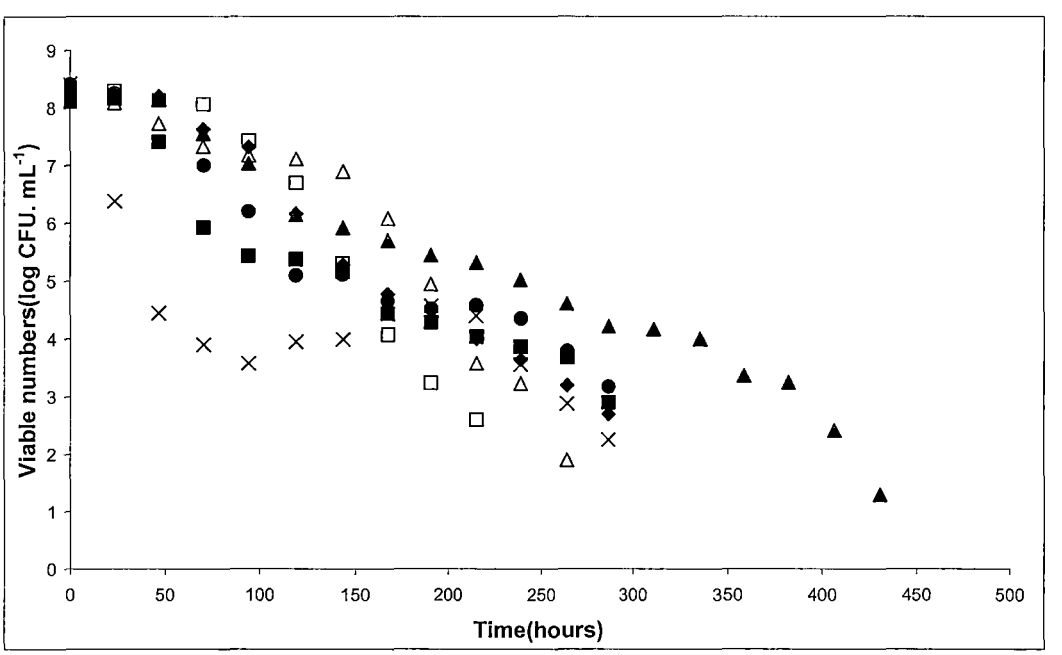

Fig B 2 (10°C) Inactivation of *E. coli* R31 (■), *E. coli* SB1 (▲), *E. coli* M23 (◆), *E. coli* MG1655 (●) and *L. monocytogenes* ATCC19115 (□), *L. monocytogenes* ScottA (x), *L. monocytogenes* Fw03/0035(Δ) in the broth model at pH 3.50 and a_w 0.900.

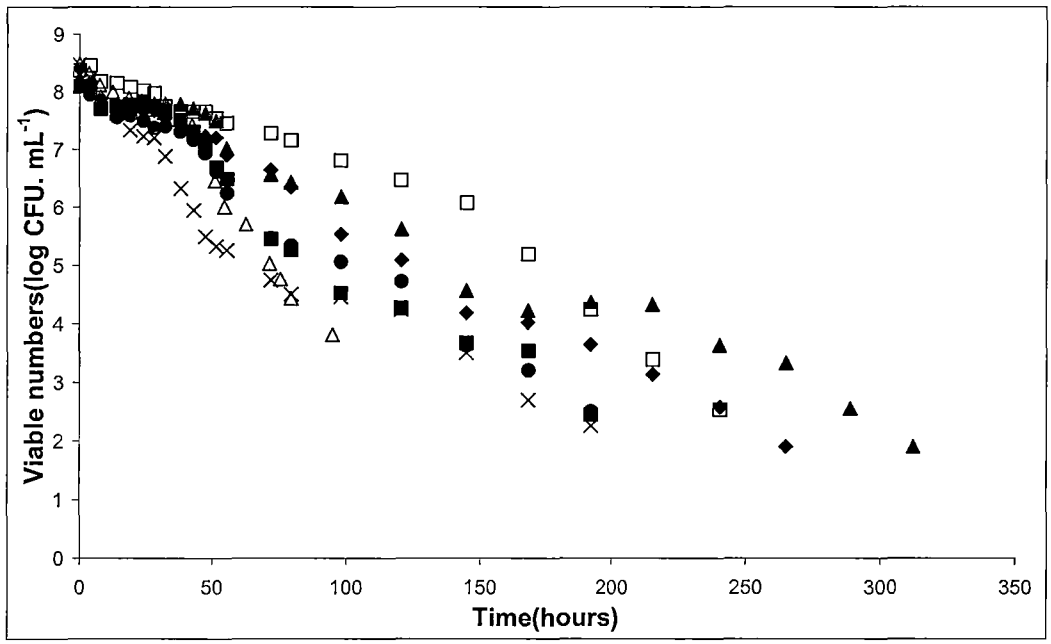

Fig B 3 (15°C) Inactivation of *E. coli* R31 (■), *E. coli* SB1 (▲), *E. coli* M23 (◆), *E. coli* MG1655 (●) and *L. monocytogenes* ATCC19115 (□), *L. monocytogenes* ScottA (x), *L. monocytogenes* Fw03/0035(Δ) in the broth model at pH 3.50 and a_w 0 900.

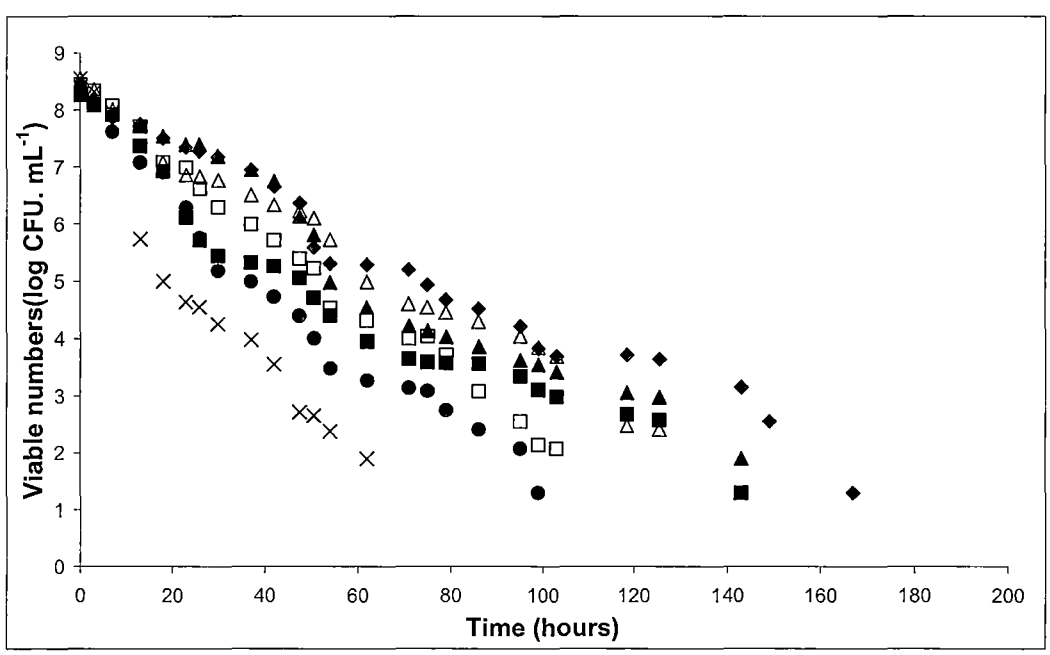

Fig B 4 (20°C) Inactivation of *E. coli* R31 (■), *E. coli* SB1 (▲), *E. coli* M23 (◆), *E. coli* MG1655 (●) and *L. monocytogenes* ATCC19115 (□), *L. monocytogenes* ScottA (x), *L. monocytogenes* Fw03/0035(Δ) in the broth model at pH 3.50 and a_w 0.900.

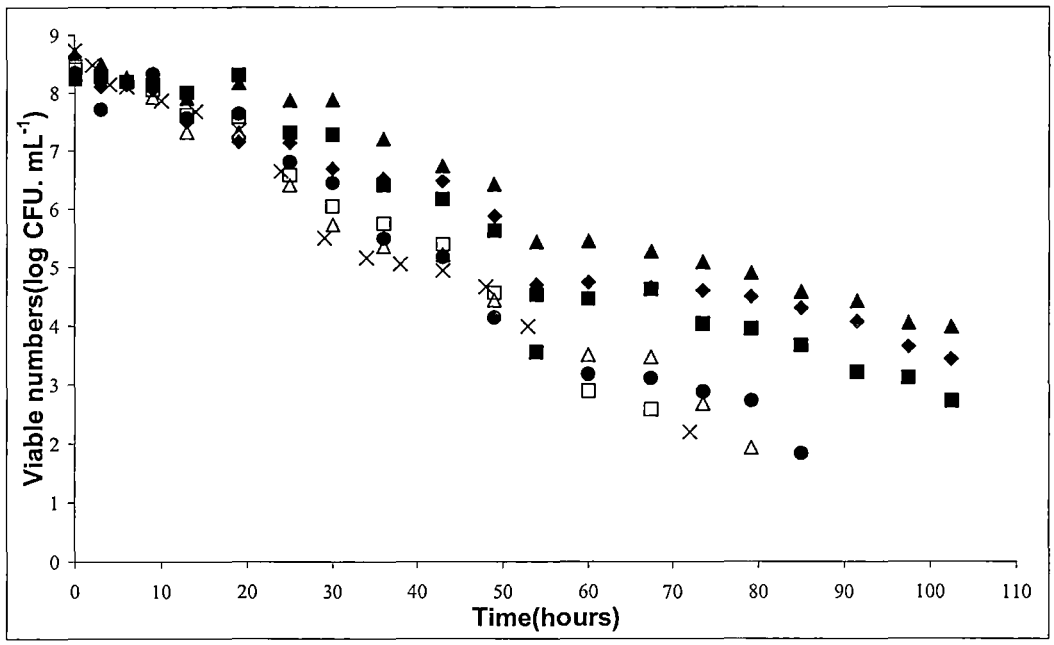

Fig B 5 (25°C) Inactivation of *E. coli* R31 (■), *E. coli* SB1 (▲), *E. coli* M23 (◆), *E. coli* MG1655 (●) and *L. monocytogenes* ATCC19115 (□), *L. monocytogenes* ScottA (x), *L. monocytogenes* Fw03/0035(Δ)in the broth model at pH 3.50 and a_w 0.900.

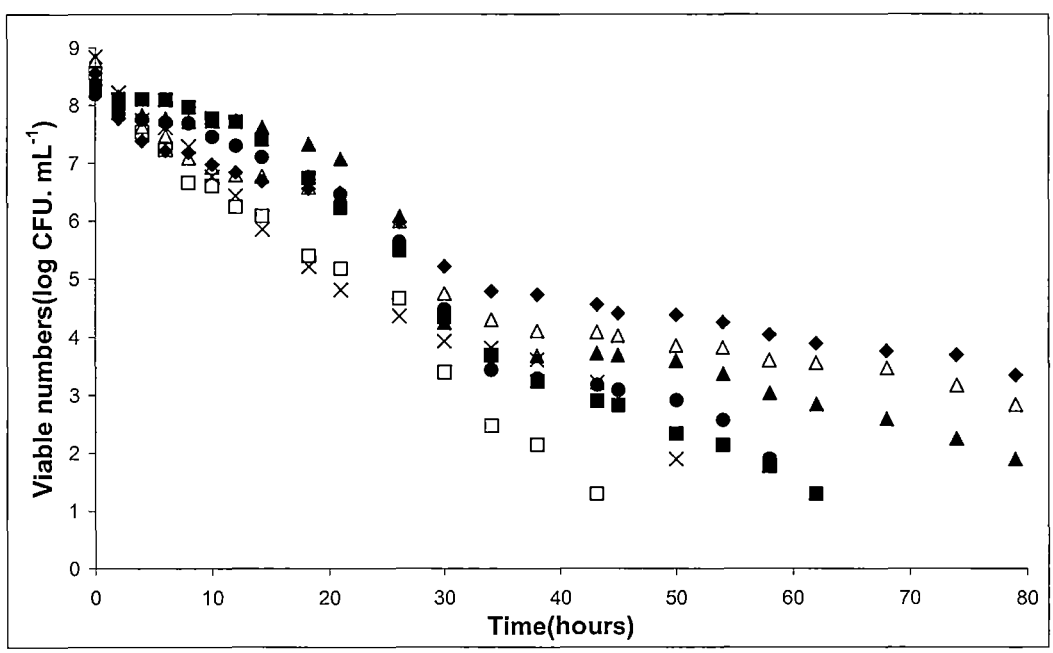

Fig B 6 (30°C) Inactivation of *E coli* R31 (■), *E. coli* SB1 (▲), *E. coli* M23 (◆), *E. coli* MG1655 (●) and *L. monocytogenes* ATCC19115 (□), *L. monocytogenes* ScottA (x), *L. monocytogenes* Fw03/0035(Δ) in the broth model at pH 3.50 and a_w 0.900

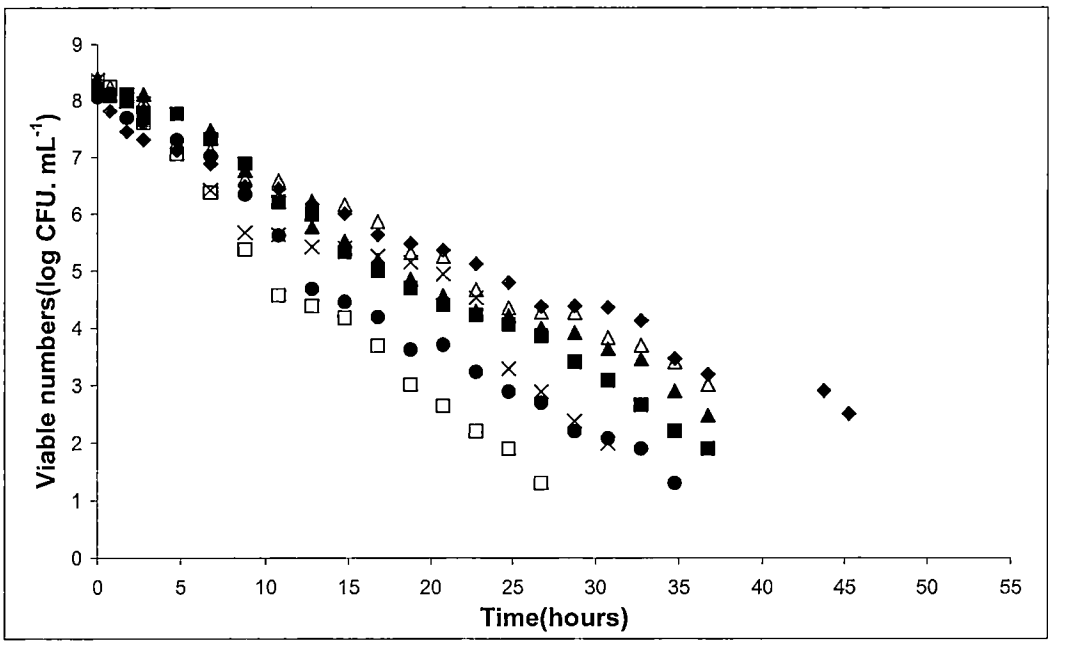

Fig B 7 (35°C) Inactivation of *E. coli* R31 (■), *E. coli* SB1 (▲), *E. coli* M23 (◆), *E. coli* MG1655 (●) and *L. monocytogenes* ATCC19115 (□), *L. monocytogenes* ScottA (x), *L. monocytogenes* Fw03/0035(Δ) in the broth model at pH 3.50 and a_w 0 900.

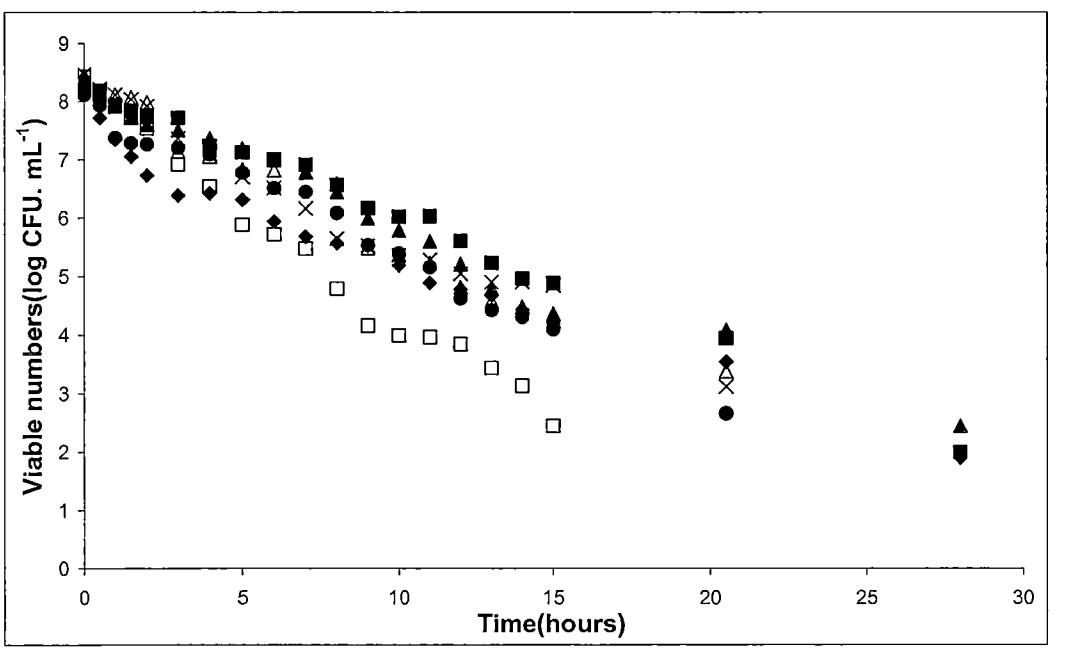

Fig B 8 (40°C) Inactivation of *E. coli* R31 (■), *E. coli* SB1 (▲), *E. coli* M23 (◆), *E. coli* MG1655 (●) and *L. monocytogenes* ATCC19115 (□), *L. monocytogenes* ScottA (x), *L. monocytogenes* Fw03/0035(Δ) in the broth model at pH 3.50 and a_w 0 900.

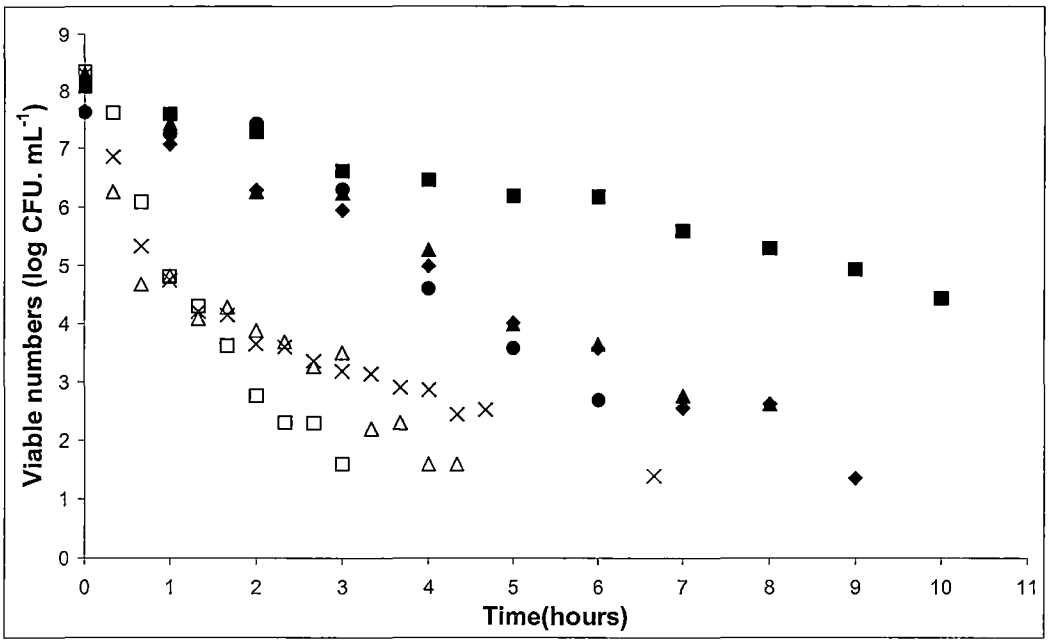

Fig B 9 (45°C) Inactivation of *E. coli* R31 (■), *E. coli* SB1 (▲), *E. coli* M23 (◆), *E. coli* MG1655 (●) and *L. monocytogenes* ATCC19115 (□), *L. monocytogenes* ScottA (x), *L. monocytogenes* Fw03/0035(Δ) in the broth model at pH 3.50 and a_w 0.900.